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Analyses of microbial communities and metabolites in Korean fermented soybean foods, meju and doenjang, and *Bacillus subtilis* pan-genome

한국의 전통 콩 발효식품인 메주와 된장의 미생물 군집 및 대사체 분석과 *Bacillus subtilis* 판지놈 분석 연구

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Abstract

Analyses of microbial communities and metabolites in Korean fermented soybean foods, meju and doenjang, and *Bacillus subtilis* pan-genome

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In this study, microbial communities and metabolites in Korean fermented soybean foods, meju and doenjang, were investigated and pan-genome analysis of *Bacillus subtilis*, known to play important roles during meju and doenjang fermentation, was also performed. In addition, I reviewed recent researches and functional properties of meju and doenjang in chapter 1.

In chapter 2, bacterial communities and biogenic amine contents in ten traditional and three commercial doenjang products were investigated. Analysis of bacterial community using pyrosequencing showed that the genera *Bacillus*, *Pseudomonas*, *Enterococcus*, and *Staphylococcus* were dominated, although the bacterial communities were different depending on the sample. Lactic acid bacteria (LAB) such as *Weissella*, *Lactobacillus*, *Leuconostoc*, and *Tetragenococcus* were also detected with relatively high abundance in some doenjang samples. Overall, the levels of biogenic amines in traditional doenjang were much higher than those in commercial doenjang. In particularly, the levels of biogenic amines in samples C (547.46 mg/kg), D (525.46 mg/kg), and F (524.19 mg/kg) exceeded the criterion recommended by FDA (500 mg/kg). A total of 432 strains were isolated from six traditional doenjang products showing three high- and tree low-histamine levels, and they were classified into seven genera and 30 species. Amylase, protease, and lipase activities of representative 13 species were tested on TSA additionally supplemented with 5% NaCl. Among them, strains belonging to *B. siamensis* and *B. subtilis* subsp. *subtilis* showed the highest enzyme activities for amylase, protease, and lipase. The features and safety of 12 strains belonging to *B. subtilis*, which is the prerequisite to use as a starter culture in Korea, was investigated. *B. subtilis* 10TDI13 was selected

as a doenjang starter based on the results. These results suggest that the strains satisfied all requirement that can be used as appropriate starter candidates for doenjang fermentation with high quality and safety.

In chapter 3, *B. subtilis* represents the best characterized member of the Gram-positive bacteria. In this study, we analyzed the taxonomic status and molecular phenotypes using the whole genome sequences of 99 *B. subtilis* and its close relative strains including strain 10TDI13, which was isolated from Korean traditional doenjang, available in GenBank. Although *B. subtilis* that consists of three subspecies (*B. subtilis* subsp. *subtilis*, *B. subtilis* subsp. *spizizenii*, and *B. subtilis* subsp. *inaquosorum*) is not phenotypically distinguishable and shares very high 16S rRNA gene sequence similarities. However, average nucleotide identity (ANI) values showed that the 99 strains can be split into at least 8 different phylogenetic lineages. The phylogenetic tree based on 308 core gene showed that three (lineage VI, VII, and VIII) of these lineages belonged to '*B. atrophaeus*', '*B. amyloliquefaciens*', and '*B. velezensis*', respectively. In addition, the rest five lineages shared low ANI values with other lineages, indicating that they should be reclassified into a different species from *B. subtilis*. The pan-genome analysis of 91

genomes belonging to *B. subtilis* was found unique genes exclusively presented in each lineage, which can be used as maker gene to distinguish each lineage.

In conclusion, the pan genome data obtained in this study demonstrated that three *B. subtilis* subspecies (*subtilis, inaquosorum*, and *spizizenii*) are clearly distinguished, supported that these five lineages could be classified as separate species.

In chapter 4, I prepared meju samples using a Korean traditional method and inoculated *A. oryzae* SNU-HR, an aflatoxin- and CPA-non-producer strain isolated from industrial koji, and then compared their qualities and functionalities. Bacteria such as *Bacillus*, unclassified *Bacillales*, *Staphylococcus*, *Leuconostoc*, and *Weissella*, were mainly detected and fungal community of doenjang samples included *Aspergillus*, unclassified *Microascaceae, Eurotium, Gibberella,* and *Mucor.* The community analysis revealed that *Bacillus* and *Aspergillus* were predominant in meju regardless of the use of starter. The meju inoculated with *A. oryzae* SNU-HR showed difference in pH, water contents, bacterial and fungal communities, and metabolites. The growth of facultative anaerobic bacteria in early fermentation

period may affected to the pH decrease and the decline of bacteria using organic acids led to that the pH of TMA is lower than TMC. The addition of *A. oryzae* SNU-HR led to increase the concentration of most amino acids including alanine, asparagine, aspartate, glutamate, glutamine, and glycine. This study suggests that the use of *A. oryzae* SNU-HR can reduce the fermentation time by producing metabolites within comparably short times and produce safe and high-quality doenjang.

Keywords: Meju, doenjang, *Bacillus subtilis*, pan-genome, *Aspergillus oryzae*, bacterial community, metabolites

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Chapter I.

Literature Review

I-1. Meju

Meju, fermented soybean brick, is the essential ingredient for the manufacture of traditional Korean fermented soybean foods including doenjang (soybean paste), ganjang (soy sauce), and gochujang (hot pepper paste). Soybean, the main ingredient of meju, contains basic nutritive constituents, such as lipids, vitamins, minerals, free sugar. In addition, it also has high essential amino acids, essential fatty acid, isolavone, flavonoid, saponins and peptides, which contributes to a wide range of health benefits (Faraj, et al. 2004, Friedman, et al. 2001, Sanjukta, et al. 2016). Therefore, soybean fermented foods have been used as main protein sources in Asian. During fermentation, soybean protein, carbohydrates, lipid are decomposed into peptide, amino acid, free sugar, and aroma constituent and these substances affect to doenjang quality.

I-1-1. Manufacturing process of meju

The manufacturing skill of traditional meju has been passed down from generation to generation by empirically and domestically without a system for sterilization or aseptic fermentation (Park, et al 2014). Although the manufacturing method of meju are various depending on region and purpose, traditional meju is generally classified into two types of meju in Korea. One is the doenjang-meju, which is for the preparation of doenjang and ganjang, and the other is gochujang-meju, which is for the preparation of gochujang. Moreover, doenjang-meju is divided into traditional meju and commercial meju depending on the ingredients.

Traditional meju: Traditional meju is manufactured in home by different process depending on the region. Meju is naturally fermented, therefore, its physicochemical and functional properties vary due to differences in soybean, geographical location, microorganisms, and fermenting time (Lee, et al. 2010). Likewise, the conditions of traditional meju fermentation are so various that it is difficult to standardize meju quality and make it impracticable to commercialize.

Generally, soybean is used as the only ingredient in Korean traditional meju process. The preparation process of the traditional meju is that soaked soy bean are boiled,

mashed, shaped the soybean into blocks by hands, and allowing it to ferment at room temperature by hung for about 6-8 weeks under the natural environment conditions (Kim, et al. 2011, Jung, et al. 2014).

Although most soybean lumps generally have rectangular shape, the shapes of the lumps are very different depending on location; globular shaped (Uljin, Gyeongbuk), cone-shaped (Seongnam, Gyeonggi), toroidal-shaped (Sunchang, Jeonbuk).

Commercial meju: Koji, which is Japanese soybean fermentation starter, is made of wheat flour by inoculating the pure cultures of *Aspergillus oryzae*, and then fermented for 2~3 days. Koji is widely used to produce fermented soybean products, especially commercial fermented soybean products (Kim, et al. 2010, Shukla, et al. 2014). Generally, rice starch and barley starch are used to improve the growth of starters, and those are decomposed into glucose and maltose by starter. The use of koji in process of meju is more convenient than the traditional method and make it easy to maintain uniform quality and to apply the mass production.

In addition, Koji can curtail meju fermentation period with proteases, amylases, and lipases produced by fungi. However, the fermented products prepared by koji have

relatively lower metabolites, which could effect on qualities and physiological functionalities, than traditionally produced fermented foods (Park, et al. 1990).

I-2. Doenjang

Doenjang is one of the representative Korean traditional fermented soybean food and it has been traditionally manufactured from meju. Doenjang is usually consumed in some cases for thousands of years as an ingredient or seasoning in various sauces, soups, or stews. Doenjang recently aroused public interest, because of the general preference for their excellent taste and nutritional value over Korean. It is regarded as a good source of essential amino acids and fatty acids, particularly for people on a grain and vegetable based diet. Koreans eat doenjang mostly in form of soups and sometimes eaten with vegetables, and the average amount consumed daily is 8.8 grams (Health Technology portal service, 2010). During the fermentation process, decomposing soybean by extracellular enzyme produced by the bacteria and fungi makes a variety of metabolites and they are given a unique flavor and savory doenjang taste. Hwang *et al.* measured doenjang composition. Doenjang compositions were 54.7% water, 13.8% crude protein, 8.0% crude lipid, 14.4 ml of titrable acidity, and 11.8% salt (Hwang, et al. 2008). Besides, doenjang contains free amino acids (glutamic acid, leucine, alanine, histidine, lysine, proline, and valine etc.), organic acid (lactic acid, malic acid, citric acid etc.), and fatty acids (linoleic

acid, oleic acid, and linolenic acid etc.) (Park et al. 2000). Likewise, doenjang has a variety of peptides and they showed beneficial effects on human health.

Because of the natural fermentation process of meju, the composition of microorganisms can be changed depending on the region, wheather, facility and it affects the taste, flavor, and quality of doenjang product. Therefore, it is not easy to keep the uniform quality of doenjang produced by conventional manufacturing methods. So, most of the research related to doenjang are focused on control of the microbial community using starter culture to produce standardized doenjang.

Doenjang can be divided into traditional doenjang and commercial doenjang depending on the ingredients and the process of manufacture. These two type of doenjang showed different functionality, microbial composition.

I-2-1. Manufacturing process of doenjang

Doenjang can be divided into traditional doenjang and commercial doenjang depending on the ingredients and the process of manufacture.

I-2-2. Traditional doenjang: the fermented meju, salt, and water are placed in pottery and this mixture is fermented for at least 40-60 days to make doenajng. Traditionally prepared meju is used as the main ingredient for the preparation of

traditional doenjang. Doenjang is made by traditional meju with 18% sodium chloride water for one month at room temperature. After one month fermentation, the fermented sample was separated in liquid and solid phases. The solid phase was stored in an earthenware pot for fermentation for 6 months - 1 year in order to obtain doenjang product.

I-2-3. Commercial doenjang: according to rapid urbanization and industrialization of society, the process of fermented soybean is structured to reduce fermentation time and be convenient to prepare, as well as, improve the quality and functionalities using. Among many strategies improving doenjang quality, the method using meju starter such as *B. subtilis*, koji inoculated *A. oryzae* are best applied to manufacture fermented soybean products. To enhance enzyme activities of starters, additional grains such as wheat and bar-ley and other flavor enhancing materials are used in sometimes. In addition, the commercial doenjang has to focus on large-scale production methods and produced under more controlled conditions in order to obtain more standardized products.

Commercial doenjang is generally prepared as flowing methods; 1) the preparation Koji [(i) Soybean are washed and soaked in tap water for 12-15 h before steaming.

(ii) Steamed soybeans were mashed, inoculated with *A. oryzae*, and then incubate for 2-3 days at room temperature] 2) Koji is mixed with the same abount of steamed and mashed soybeans 3) added salt to adjust the final NaCl concentration of 10%. 4) Fermentation for 3~6 months at room temperature.

I-3. Health benefits of doenjang

As many kinds of microorganism occurred naturally during meju fermentation, enzymatic reaction on fermentation makes small molecules, sugars, peptides, amino acids, and free fatty acids. These molecules can be converted into diverse volatiles by secondary reactions through further fermentation, which lead to distinctive flavor compounds of *doenjang* (Cha, et al. 2012). During soybean fermentation, not only tastes and flavors but various flavonoids, vitamins, phytoestrogens, unsaturated fatty acids, and functional peptide are made and aglycone such as daidzin, genistin converted to daizein, genistein which were bioactive substance, supporting that previous study showed that the GABA (γ-aminobutyric acid) was increased with the decrease glutamic acid and GABA precursor (Jo, et al. 2011). These substances demonstrate some health benefits strong antimutagenic activities against various carcinogens and mutagens, and also exhibit antioxidant, anticancer activities, fibrinolytic activity, immune-modulatory and preventive effects on cardiovascular disease (Abdou, et al. 2006, Candela, et al. 2006). In addition, as ripening period increased, its taste and principal components of soybeans are easy to use are increased. Korean fermented soybean foods also have similar functional activities.

Many study reported that traditional doenjang have higher functional activities than other traditional Korean fermented soybean products such as commercial doenjang, chungkukjang, and miso. Increased functional activities probably due to the differences in microbiological characteristics of Meju. (Cheigh, et al. 1993)

I-3-1. Fibrinolytic activity

According to the World Health Organization (WHO) report, 17 million people die due to cardiovascular diseases (CVD) every year (WHO, 2016). Intravascular thrombosis, the formation of a clot of blood in a blood vessel, is one of the main causes of a variety of CVDs. The fibrin clot formation at the site of an injury to the wall of a normal blood vessel is an essential process to stop blood loss after vascular injury. The formation of a hemostatic clot requires thrombin-mediated cleavage of fibrinogen to fibrin Clots formed in the presence of low thrombin concentrations are composed of thick fibrin fibers and are highly susceptible to fibrinolysis; while, clots formed in the presence of high thrombin concentrations are composed of thin fibers and are relatively resistant to fibrinolysis (Wolberg, 2007, Wolberg, et al. 2008). In general, fibrin clot are naturally decompose by fibrinolytic enzyme such as plasmin in human body. But, accumulation of fibrin in the blood vessels under an unbalanced

situation due to some disorders, the clots are not hydrolyzed, and it cause thrombosis, leading to myocardial infarction, other cardiovascular diseases, or sometime result in death. Many researcher reported that traditional Korean soybean foods, meju and deonjang, have rich in fibrinolytic enzyme and isolated many strains, which has that activity, and successively discovered the enzyme. Among the strains, most strains were the genus *Bacillus*, which is known as the dominant genus in meju and deonjang. Therefore, microbial fibrinolytic enzymes and food containing this activity have attracted much more attention than typical thrombolytic agents (t-PA and urokinase), which have undesirable side effects as well as expensive. (Peng, et al. 2005, Mine, et al. 2005, Singh, et al. 2014).

I-3-2. Anticancer effects

Many studies reported that fermented soybean food contain higher level of bioactive compounds and exhibit stronger anti-oxidative and anticancer activities than unfermented ones (Gibbs, et al. 2004, De, et al. 2006). These functions are also correlated with fermentation period. Jung showed that 24 month fermentation doenjang exhibited a 2-3 fold in antitumor effects on scarcoma- 180- injected mice and antimetastatic effects in colon 26-M 3.1 cells in mice by decreasing tumor

formation and increasing natural killer cell activity in spleens and glutathione S-transferase activity in livers of mice compared with the 3 or 6 month fermented doenjang **(**Jung, et al. 2006). When Hwang used 24 and 48 month fermented doenjang extracts to examine the anticancer and apoptotic effect in AGS human gastric adenocarcinoma cells, the treatment of 24 month fermented doenjang extract was more effective on inhibiting AGS cancer cell growth than 12 month fermented doenjang extract (Hwang, et al. 2005). Kwon and shown have reported that the extracts from ripened for 3 years deonjang have strong anticancer activities in water fractions for human lung carcinoma in A-549 cells and human breast carcinoma MCF-7 cells compared with those for 1 or 2 years (Kwon, et al. 2004).

Doenjang prepared with bamboo salt showed definitely higher anticancer effect than purified salt-Doenjang on HT-29, human colon cancer cells, and this study also proved that better as ripening period increased. **(**Jung, et al. 2006, Shim, et al. 2015, Lee, et al. 2009)

I-3-3. Antimutagenic activity

Traditional *doenjang* extracts showed strong antimutagenic activities induced by aflatoxin B1, N-methyl-N′-nitro-N-nitrosoguanidine, and 4-nitroquinoline-1-oxide,

moreover, against various carcinogen/mutagens including aflatoxin B1. Moreover, the active compounds including genistein, linoleic acid, β-sitosterol glucoside, and soyasaponin in doenjang extracts are exhibited strong antimutagenic activities in the Ames test, SOS chromotest and Drosophila wing spot test (Hwang, et al. 2008, Park, et al. 2003). Although, Jang demonstrated that extensice isoflavone metabolism occurred during meju fermentation but not during aging of doenjang, leading to significant decrease of total isoflavones and comprehensive conversion of glycosides into aglycones, the traditional doenjang has relatively high level of aglycones such as genistein compared with other soy products. (Jang, et al. 2008)

I-3-4. ACE inhibitory effect

Hypertension (HTN or HT), also known as high blood pressure (HBP), is a long term medical condition in which the blood pressure in the arteries is persistently elevated. According to the Global health observatory (GHO) data, approximately one billion adults or ~22% of the population of the world have hypertension in 2014 (http://www. who. int/gho/tb/en, 2015). Detection of HTN / HBP are critically important for reducing the risk of heart attacks and strokes. *Doenjang* is known to contain di-peptide as arginine-proline, which has the abilities to produce an ACE inhibitory

effect. Consumption of doenjang results in lower the risk of heart disease. Other peptide substances of doenjang have blood pressure depressant abilities (Shin, et al. 2015). Regular consumption of the Korean fermented soybean foods by hypertensive and Type 2 diabetic patients results in favorable changes in cardiovascular risk factors (Jung, et al. 2014) and reduction of hypocholesterolemic effect (Lim, et al. 2014). ACEs inhibitory peptides derived from food proteins are used for treating hypertension (Jakubczyk, et al. 2013).

I-3-5. Obesity

Previous studies were compared the antiobesity effect of doenjang with that of non-fermented soybeans using animal model. Doenjang showed lowered fatty acid synthase (FAS) activity and elevated carnitine palmitoyltransferase (CPT)-1 activity in liver tissue (Kwak, et al. 2011). Not only that, body weight gain and hepatic TG and cholesterol levels were significantly lower in doenjang compare with non-fermented soybean. The ability of doenjang to control obesity is increases as prolonging the fermentation period. Fermented paste have been reported to effectively reduce the body weight and body fat, improve the glucose metabolism, and enhance the antioxidant defense stats in mice under high fat diet condition

(Chung, et al. 2014). Therefore, taking of traditional doenjang could be effective on reducing abdominal fat and weight control in obese people (Cha, et al. 2012).

I-3-6. Other functions

Except for Fibrinolytic activity, anticancer activity, antimutagenic activity, ACE inhibitory effect, and obesity, doenjang have various functions, beneficial to human health. For instance, the consumption of doenjang showed that not only decrease the E-antibodies produced from the antigen (Lee, et al. 1997), but also enhance immune function such as preventing allergies (Jang, et al. 2008). In addition, taking of the doenjang is effective to prevent and cure lifestyle related disease and prevent to osteoporosis, arteriosclerosis, strokes, and dementia. (Kim, et al. 2010).

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Chapter II.

A survey of traditional- and commercial-doenjang products focusing on bacterial communities and biogenic amines

II-1. Introduction

Doenjang is widely used as an essential condiment to enhance flavors as well as protein rich food in Korea for more than a thousand years. Doenjang can be sorted into traditional doenjang and commercial doenjang depending on the ingredients and the process of manufacture. Traditional doenjang is prepared by mixing fermented soybeans (meju) with 18% brine in a pottery, followed by fermenting for approximately two months then separating it into liquid and solid phase (Jo, et al. 2011). The solid phase is subsequently mashed and stored in a porcelain pot for fermentation for 6 month-1 year in order to obtain doenjang product. In contrast, the process using meju starter such as *Bacillus subtilis* or *Aspergillus oryzae* is adapted in commercial doenjang (Lee, et al. 2012). In general, Koji is prepared as follow; steamed wheat is inoculated with *A. oryzae*, and then incubate for 2-3 days at room temperature. The koji is mixed with the same amount of steamed soybeans and approximately 10% NaCl, and then store for fermentation at room temperature for 3-6 months, which is shorter than the period of fermentation in traditional doenjang. In commercial doenjang process, the use of koji can curtail meju fermentation period with extracellular enzymes generated by fungi. Therefore, the mass production is

feasible to commercialize with uniform quality. On the other hands, traditional doenjang is difficult to standardize the quality because physicochemical and functional properties of meju, which is major ingredient of doenjang production, are vary depending on microorganisms derived from ambient air, dried rice straw, and the facility. More varied microorganisms are participated in traditional doenjang fermentation and produce various kinds of metabolites that affect the flavors and tastes of doenjang, therefore some Korean prefer to traditionally manufactured doenjang.

The consumption of doenjang has been gradually increased because of its excellent nutritional value and beneficial impact on human health such as anticancer activity, antimutagenic activity, and high blood pressure depressant ability (Shim, et al. 2015, Park, et al. 2003, Shin, et al. 2015). Although doenjang has so many advantages, it sometimes threatens human health by toxic substances such as mycotoxin, aflatoxin, and BAs produced by microorganisms (Park, et al. 2003, Shukla, et al. 2010). In particular, some microorganisms are produced decarboxylase so that it is capable of producing biogenic amines. Excessive consumption of these amines can be of health concern as BAs can generate different degrees of diseases determined by their action on nervous, gastric and intestinal systems and blood pressure in human organism

(Suzzi, et al. 2003). In addition, BAs are considered as carcinogens because of their ability to react with nitrites to form potentially carcinogenic nitrosamines (Ö nal, et al. 2007). Therefore, assessment of BAs in doenjang is essentially important to secure the quality and safety.

The aim of this study was to investigate the bacterial community and biogenic amine level in traditional doenjang and commercial doenjang. The bacterial community was investigated by pyrosequencing, culture independent method, and biogenic amine level was determined by HPLC in doenjang products. In addition, the bacterial strains were isolated on the TSA media containing 5%, 10%, and 15% NaCl. Among them, the doenjang starter candidates were screened by amylase, protease, and lipase activities, and then their safety and functional properties were also tested to evaluate them as potential starter to improve doenjang quality.

II-2. Materials and Methods

II-2-1. Doenjang sample collection and measurement of pH and titratable acidity

Ten traditional doenjang samples were collected from different manufacturers at folk villages of Sunchang region in Jeonla province and three commercial products were purchased from a local market in Seoul, Korea and immediately stored at -20℃.

For pH measurement, 90 ml of deionized distilled water was added to 10 g of doenjang samples, homogenized. The pHs of the samples were measured using a digital pH meter (Orion 3-Star Plus pH Meters, Thermo Scientific, USA). The acidity of doenjang samples was measured by the AOAC method. The required volume of NaOH to neutralize 90ml doenjang was measured, and then the volume of NaOH was calculated into the amount of lactic acid of the doenjang sample.

Total acidity (%) = amount 0.1N NaOH used (ml) x concentration of NaOH solution (N) x constant of organic acid equivalent to 1ml of 0.1N NaOH solution (in the case of lactic acid = 0.009) x 100g

II-2-2. Genomic DNA extraction and pyrosequencing

For the phylogenetic analysis of *Bacteria* in meju, microbial cells of ten traditional doenjang and three commercial doenjang samples were disrupted using a bead-beating method (Yeates, et al. 1998) and then total genomic DNA was extracted using the QiAamp DNA stool mini kit (Quiagen, USA) according to the manufacturer's instructions. To analyze the bacterial community, the V1/V3 region of the 16S rRNA gene sequences in the genomic DNA were amplified, sequenced, and analyzed as previously described with some modified (Kim, *et al.* 2011). To amplify the bacterial 16S rRNA gene fragments, 20 ng of purified DNA (confirmed NanoDrop 1000 spectrophotometer) was amplified. The V1-V3 hyper-variable regions of the bacterial 16S rRNA gene (Kim, et al. 2012) were amplified with the primer pair 27F (5'-adaptor A-AG-27 F-3') / 518R (5'-adaptor B-X-AG-518R-3') containing ten base sample-specific barcode sequences (Table II-1) and common linker AG sequences in the 5' end (Hamady, et al. 2008). Thermocycling was conducted in a C1000 Thermal Cycler (Bio-Rad, CA, USA) under the following conditions: initial denaturation at 94℃ for 3 min; 30 cycles of denaturation at 94℃ for 45 s, annealing at 58℃ for 45 s, and extension at 72℃ for 1 min; and a final extension at 72℃ for 10 min. The quality of the amplified PCR products was verified by electrophoresis

in a 1% agarose gel and purified using a PCR purification kit (Bioneer, Korea). An equal quantity (50 ng) of each PCR amplicon tagged with the sample-specific barcode sequences was pooled. The pooled DNA was amplified by emulsion PCR before sequencing by synthesis using the massive parallel pyrosequencing protocol (Margulies, et al. 2005) and then sequenced using a 454GS-FLX titanium system (Roche, Germany) at Macrogen (Korea).

The sequences were then analyzed using the Mothur program (ver. 1.10.0) (Scholoss, et al. 2009). The pyrosequencing reads were sorted to the specific samples based on their unique barcode sequence, after which the barcode, linker and both forward and reverse primer sequences were trimmed form the original sequence.

Table II-1. List of adapters, 16S rRNA primers, and barcode sequences in the PCR primer sets used in this study

Name	Sequence (5'->3')	Reference
Adapter sequence		
A adapter sequence	CCATCTCATCCCTGCGTGTCTCCGACTCAG	Haas et al., 2011
B adapter sequence	CCTATCCCCTGTGTGCCTTGGCAGTCTCAG	Haas et al., 2011
16S rRNA primers		
27F	GAGTTTGATCMTGGCTCAG	Kim, et al. 2012
518R	WTTACCGCGGCTGCTGG	Kim, et al. 2012
Doenjang sample		
A	ACGAGTGCGT	This study
B	ACGCTCGACA	This study
C	AGACGCACTC	This study
D	AGCACTGTAG	This study
E	ATCAGACACG	This study
F	ATATCGCGAG	This study
G	CGTGTCTCTA	This study
H	CTCGCGTGTC	This study
I	TCTCTATGCG	This study
J	TGATACGTCT	This study
K	CATAGTAGTG	This study
L	AGCACTGTAG	This study
M	ATCAGACACG	This study

II-2-3. Isolation and identification of isolates strains from doenjang

To select doenjang starter candidates, six samples based on histamine level of traditional doenjang were used. For isolation of bacteria, 25 g of each doenjang sample was mixed with 225 ml buffered peptone water (Oxoid) and homogenized for 2 min in a stomacher (Easymix BLENDER, France). After homogenization, 1 mL aliquots of the sample were serially diluted in 9 mL of sterile buffered PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.2), and 0.1 mL of the diluted suspension was plated on Tryptic Soy Agar (TSA, Difco, USA) and TSA containing 5, 10, and 15% NaCl (w/v). After the plates were incubated at 30℃ for 1, 3, 5 days, respectively, over 20 distinctive single colony based on differences in morphology, growth characteristics was selected. The selected colonies were transferred on the same type of agar medium for isolation. Isolates were stored until needed at -80℃ with 20% sterile glycerol.

Genomic DNA of isolates was extracted using G Spin total DNA extraction kit (Intron Biotech). Amplification of the 16S rRNA gene was performed with eubacterial universal primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (Lane, 1991) in a C1000 thermal cycler (Bio-Rad) with 25 μl volume. the cycling regime was as follows: 94°C for 5

min (1 cycle); 94℃ for 45 s, 55℃ for 45 s, and 72℃ for 45 min (30 cycles); and 72℃ for 10 min (1 cycle). The PCR amplicons were purified using a PCR purification kit (Bioneer, USA) and sequenced using a custom service provided by Macrogen Inc. (Seoul, Korea). The 16S rRNA gene sequences were BLASTN-analyzed in the National Center for Biotechnology Information and EzTaxon server 2.1 databases to search closely related sequences (Chun, et al. 2007).

II-2-4. Analysis of biogenic amine level of doenjang and Statistical analysis

Biogenic amine reagents including agmatine sulfate (AGM), cadaverine dihydrochloride (CAD), tryptamine hydrochloride (TRP), putrescine dihydrochloride (PUT), histamine dihydrochloride (HIS), tyramine hydrochloride (TYR), spermidine trihydrochloride (SPD), spermine tetrahydrochloride (SPM), 2-phenylethylamine (PHE), benzoyl chloride, and acetone were purchased from Sigma–Aldrich. Each reagent was dissolved in 0.1 M HCl solution and the final concentration of each amine (free base) was 1.0 mg/ml. These solutions were stored at 4℃ until the use.

Extraction of BAs from Doenjang and determination of BAs were determined by a liquid chromatographic method as described by Hwang et al. (1997) with some

modified. To extract BAs, 25 mL of 0.1 N HCl was added to 10 g of each Doenjang samples, and the mixture was homogenized for 15 min. The homogenate was centrifuged at 3000 × g at 4℃ for 15 min. The supernatant was collected and the residue was extracted twice with the same volume (25 mL) of 0.1 N HCl solution. All collected supernatants were combined and the final volume was adjusted to 50 mL with 0.1 N HCl acid. Two milliliter of each extracted sample or mixed standard amine solution was mixed with 1 ml of 2 M sodium hydroxide, followed by 10 μl of benzoyl chloride, the solution was mixed by using a vortex mixer and was allowed to stand at 30℃ for 40 min. The benzoylation was stopped by adding 2 ml of saturated NaCl solution, and the solution was extracted with 3 ml of diethyl ether. After centrifugation, the upper layer was transferred into new tube and evaporated to dryness in a stream of nitrogen. The residue was dissolved in 1 ml of methanol and 10-μl aliquots were injected for HPLC analysis. The supernatant was filtered through 0.45 μm × syringe filters (Millipore, USA). The filtered supernatant was kept at -25℃ until assayed by HPLC.

Biogenic amine level was determined using DIONEX Ultimate 3000 high-performance liquid chromatography (HPLC) equipped with Ultimate 3000 monitored by UV detector at 254 nm, and a Acclaim 120 C-18 column (250 mm x

4.6 mm, 5μ, Thermo Fisher Scientific Inc., Sunnyvale, CA, USA) for chromatographic separation. The gradient elution program began with 50:50 acetonitrile: 0.1 M ammonium acetate (v/v) at a flow rate of 1 mL/min, followed by a linear increase to 90:10 acetonitrile: 0.1 M ammonium acetate for 19 min, which was decreased to 50:50 over the final 2 min. All experiments were conducted three times on independent samples prepared.

Data collected were analyzed by one-way analysis of variance (ANOVA). The Tukey's test was used for mean comparison when a significant variation was found by the ANOVA test. The significance of results was at $P < 0.05$. The software used was IBM SPSS Statistic version 21 (2005).

II-2-5. Evaluation of protease, amylase, and lipase activities

The proteolytic and amylase activity of isolated strains were evaluated by the quantitative disc diffusion method. Each isolated strains was cultured in 5 ml TSB (Difco, USA) at 30℃, and cell-free supernatant was obtained from the cultures by centrifugation (13200 rpm, 15 min). For confirm protease activity, Cell-free supernatant (20 μl) was loaded on paper disk on TSA agar (Difco, USA) containing 1.5% bovine skim milk powder (Difco, USA) and the protease activity was evaluated

based on clear zones forming around the paper disks after a 24h incubation at 30℃. For investigation of amylase activity, starch agar plates containing 1% soluble starch, 0.2% yeast extract, 0.1% K_2HPO_4, 0.15% 160 $MgSO_4 \cdot 7H_2O$, 7.5% NaCl, and 1.5% agar were prepared, and the 20 μl of meju supernatant was dropped onto the starch agar plates and the plates were grown at 37℃ for 24 h. Amylase activity was assayed using Gram's iodine solution after a 24-h incubation at 37℃. For the determination of lipase activities, 1% tributyrin was added to tributyrin agar (Sigma) and emulsified by sonication before autoclaving.

II-2-6. Fibrinolytic activity

Selected strains were cultured in Tryptic Soy Broth (TSB). The supernatant of culture was used as a crude enzyme. Analysis of fibrinolytic activity was conducted by the modified fibrin plate method (Choi, et al. 2001). Fibrinogen (10 mL of 0.6%) (Sigma-Aldrich, St. Louis, MO, USA) solution in a 10 mM sodium phosphate buffer (pH 7.4) was mixed with the same volume of 2%(w/v) agarose solution and 20 μL of thrombin (250 units/mL) (Sigma-Aldrich) in a petri dish. The solution was left for 1 h at room temperature to form a fibrin clot layer. 20 μL of supernatant was applied to a fibrin plate and incubated at 30℃ for 18 h. The same volume of plasmin solution

(1 NIH unit/mL) (Sigma-Aldrich) was also incubated on a fibrin plate as a positive control for fibrinolytic activity.

II-2-7. Antibiotic susceptibility test

Antibiotic susceptibility test were performed using the disk diffusion method according to the guidelines of the Clinical and Laboratory Standard Institute (2007). The cultures of 30 starter candidate strains were diluted to a 0.5 McFarland turbidity standard (bioMerieux, Marcy l'Etoile, France), and then 100 μl was spread onto Mueller-Hinton agar plates (Oxoid, Basingstoke, Hants, UK) to form an even lawn of bacteria. Sterile paper disks were placed on the surface of each plate and incubated at 37°C for 24 h. Sixteen types of antibiotic disks containing ampicillin (10 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), lincomycin (15 μg), linezolid (10 μg), neomycin (30 μg), oxaxillin (1 μg), penicillin G (10 μg), rifampicin (10 μg), streptomycin (10 μg), sulphamethoxazolec (25 μg), tetracycline (30 μg), and vancomycin (30 μg) were purchased from Oxoid. All experiments were conducted three times on separate days using fresh strain cultures.

II-2-8. Evaluation of the potential production of enterotoxin of isolated strains

To evaluate of the potential production of enterotoxin of Bacillus starter candidates, PCR was carried out targeting for enterotoxin (*nheA, nheB, nheC, hblA, hblC,* and *bceT*). The primer for detection of those genes are given in Table II-2. The amplification program was as follows: 5 min initial denaturation at 95℃ followed by 30 cycles of denaturation at 95℃ for 1 min, annealing at 56℃ for 1 min and primer extension at 72℃ for 30 s. The amplifications were terminated after a final elongation step of 5 min at 72℃.

Table II-2. PCR primers used for PCR detection of enterotoxin genes used in this study

Gene	Product	primer	Sequence F (5'->3')	Product (bp)	Annealing temp. (℃)	Reference
nheA	nonhemolytic enterotoxin gene A	nheA-F	TAA GGA GGG GCA AAC AGA AG	759	60	Ngamwongsatit (2008)
		nheA-R	TGA ATG CGA AGA GCT GCT TC			
nheB	nonhemolytic enterotoxin gene B	nheB-F	CAA GCT CCA GTT CAT GCG G	935	60	Ngamwongsatit (2008)
		nheB-R	GAT CCC ATT GTG TAC CAT TG			
nheC	nonhemolytic enterotoxin gene C	nheC-F	ACA TCC TTT TGC AGC AGA AC	618	58	Ngamwongsatit (2008)
		nheC-R	CCA CCA GCA ATG ACC ATA TC			
hblA	hemolysin BL binding component	hblA-F	GCA AAA TCT ATG AAT GCC TA	884	54	Ngamwongsatit (2008)
		hblA-R	GCA TCT GTT CGT AAT GTT TT			
hblC	hemolysin BL lytic component L_2	hblC-F	CCT ATC AAT ACT CTC GCA A	695	54	Ngamwongsatit (2008)
		hblC-R	TTT CCT TTG TTA TAC GCT GC			
bceT	encoding the bc-D-ENT enterotoxin	bceT-F	CGT ATC GGC CGT TCA CTC GG	661	55	Guinebretiere (2002)
		bceT-R	GTT GAT TTT CCG TAG CCT GGA			
cytK	encoding cytotoxin K	cytK-F	ACA GAT ATC GGG CAA AAT GC	809	54	Guinebretiere (2002)
		cytK-R	TCC AAC CCA GTT TGC AGT TC			

II-2-9. Cytotoxicity assays

To investigate the effects of the isolated strains from doenjang, cytotoxicity assay was performed using INT-407 (ATCC CCL-6) human intestinal epithelial cells. The preparation of the INT-407 cells and infection with the isolated bacterial cultures were conducted in a 96-well tissue culture plate (Nunc, Roskilde, Denmark) as previously described (Park, et al., 2006). The cytotoxicity was then determined by measuring the activity of lactate dehydrogenase (LDH) in the supernatant using a cytotoxicity detection kit (Roche, Mannheim, Germany) and expressed using the total LDH activity of the cells completely lysed by 1% Triton X-100 as 100%.

II-2-10. Quantitative evaluation of anti-pathogenic activities

The anti-pathogenic activities of bacterial strains were quantitatively evaluated. The pathogen strains, *Bacillus cereus* (ATCC27348), *Escherichia coli* (O157:H7), *Staphylococcus aureus* (KCTC 3881), and *Listeria monocytogenes* (KCTC 13064), were used as indicator strains to quantitatively evaluate the anti-pathogenic activities of the bacterial strains. Growth inhibition of four indicator strains by the bacterial test strains was tested using the top-agar method as described previously (Jeon, et al.

2016). Their quantitative anti-pathogenic activities were evaluated after a 16-48 h incubation at 37℃; the incubation time differed, depending on pathogenic bacteria.

II-2-11. Quantitative evaluation of biogenic amines production of isolated strains

The production of biogenic amines (BAs) including histamine, cadaverine, putrecine, and tyramine, by bacterial strains was examined in TSB-5% NaCl containing 0.2% of their corresponding precursor molecules (histidine, ornithine, lysine, and tyrosine) and 0.005% pyridoxal 5'-phosphate. After a 2-day incubation at 37℃, BAs in culture broths were analyzed with a HPLC system (Agilent, USA), as described previously (Mah, et al. 2003).

II-3. Results and Discussion

II-3-1. General features of doenjang samples

The general features (pH, acidity, and water content) were shown in Table II-3. The pH value of all doenjang samples was ranged from 4.5 to 5.7 and the acidity was in the 1.66-3.16 range. The moisture content of doenjang samples was differed considerably depending on the samples. The lowest level of doenjang moisture was 50.47 % in sample I, whereas the highest level was 61.43 % in sample D.

Table II-3. List of the analyzed soybean paste samples and its pHs, acidities, water contents of samples

Sample type	Sample	pH	Acidity	Moisture contents (%)
Traditional	A	5.4	1.66	55.15
	B	5.2	2.64	56.38
	C	5.7	2.73	57.22
	D	4.9	3.56	61.43
	E	5.2	3.09	55.90
	F	5.2	2.33	56.00
	G	4.9	2.77	54.67
	H	5.3	2.40	57.97
	I	5.6	1.82	50.47
	J	4.5	3.16	55.00
Commercial	K	5.3	2.12	53.26
	L	5.4	1.94	56.45
	M	5.1	2.35	54.86

Acidity (%)=[{0.009×0.1 N NaOH (mL)×F (factor of 0.1 N NaOH)}/sample (mL)]×100

0.1N NaOH factor: 1.003

lactic acid unit conversion factor: 0.009

II-3-2. Bacterial communities of doenjang samples

Bacterial communities of ten traditional doenjang and three commercial doenjnag samples were investigated by culture independent method, 454 Flex pyrosequencing (Table II-4). A total of 334,717 sequence reads of 16S rRNA gene amplicons were obtained. After barcode sorting, the number of sequences from each doenjang sample varied from 4915 to 20341. The high quality pyrosequencing reads of bacterial 16S rRNA gene sequences were classified at the phylum and genus levels. The bacterial composition was considerably different depending on the doenjang sample. The phylogenetic classification of bacterial sequences from the doenjang samples is presented in Figure II-1 and Figure II-2. At the phylum level of bacteria, *Firmicutes* was a dominant in nine samples except for A, C, E, and H samples, which have high relative abundance of *Proteobacteria* as dominant regardless of doenjang prepared types. *Actinobacteria* and cyanobacteria were also detected with minor proportion.

At the genus level of bacteria, it was shown that 13 deonjang samples have various species but their microbial community structure were quite simple. The genera *Bacillus, Pseudomonas, Enterococcus, Enterobacter, Staphylococcus, Leuconostoc*, and *Tetragenococcus* were represented the bacterial communities in doenjang samples. The ‘others’ are composed of the genus groups showing a percentage of

sequencing reads < 1.0% of the total bacterial sequences in all samples. In sample A, E, and H, 62.1 %, 63.5 %, and 53.4% of the sequences were attributed to *Pseudomonas*, whereas only 0.0-1.7 % were assigned to the same genus in other samples. Although the genus *Bacillus* is considered as the main species, which led the doenjang fermentation, only sample D (83.6 %) and M (82.9 %) of total thirteen samples were dominated by the genus. This result was generally in accordance with those demonstrated by previous study (Nam, et al. 2012). In sample I and L, the genus *Bacillus* detected 22.6 % and 29.5 %, however, less than 10 % were confirmed in other samples. Interestingly, LAB such as *Leuconostoc, Enterococcus, Tetragenococcus, Enterobacter, Weissella* were detected with relatively high abundance in some samples and LAB population completely differed depending on the sample. In sample B and F, *Enterococcus* were absolutely dominant with 67.2 % and 67.5 %, respectively, nevertheless the genus were not detected in four samples (sample J, K, and L). Because doenjang have generally 15-18% NaCl concentration, halophile and salt tolerance bacterium such as *Tetragenococcus, Oceanobacillus,* and *Halomonas* can grow in doenjang environment. In twelve of thirteen samples except for sample L, *Tetragenococcus* were detected ranged from 0.1 % to 39.6 %. In contrast, *Oceanobacillus* and *Halomonas* were detected only in two samples

(sample G and J). The safety of traditional doenjang has been an issue by several researcher (Lee, et al. 2012) as the meju, which is the main ingredient for doenjang, is spontaneously fermented in a process involving various microorganisms derived from the surrounding environment such as air and rice straws. Among varied microorganisms detected from doenjang, several species and strains of the genus *Staphylococcus* and *Enterobacter* recognized as pathogenic and cause opportunistic infections in Immunodeficiency human. In this study, *Enterobacter* were detected with 35.2 % in one sample C, while *Staphylococcus* were detected in all doenjang samples (0.1-55.5 %). Although some previous studies insisted that the most *Staphylococcus* founded from traditional food is coagulase negative *Staphylococcus* (CNS), but the safety of traditional doenjang and involved microorganisms should be systematically confirmed.

Table II-4. Data summary of pyrosequencing

Sample type	Sample	Total reads	number of reads analyzed	Average length (bp)	OTUs[(a)]	Chao	ACE	Shannon
Traditional doenjang	A	15633	7916	444	33	38.25	39.03	1.14
	B	11239	4915	449	38	98.00	82.95	1.23
	C	13347	7412	433	30	33.00	32.49	1.83
	D	17899	11844	374	47	52.25	51.49	1.98
	E	38451	9488	248	52	55.00	57.53	1.73
	F	35277	6176	292	25	39.00	39.59	0.89
	G	18401	4562	418	57	72.00	63.59	2.31
	H	25782	10634	413	50	59.43	61.08	1.64
	I	35966	10753	394	60	63.75	63.06	2.66
	J	24018	8973	421	57	58.67	59.36	2.08
Commercial doenjang	K	46855	20341	376	34	55.00	39.12	1.12
	L	27612	14252	441	28	35.00	36.05	1.33
	M	24237	11500	446	35	37.50	41.07	1.59

[a)]The operational taxonomic units (OTUs) were defined with 3% sequence dissimilarity.

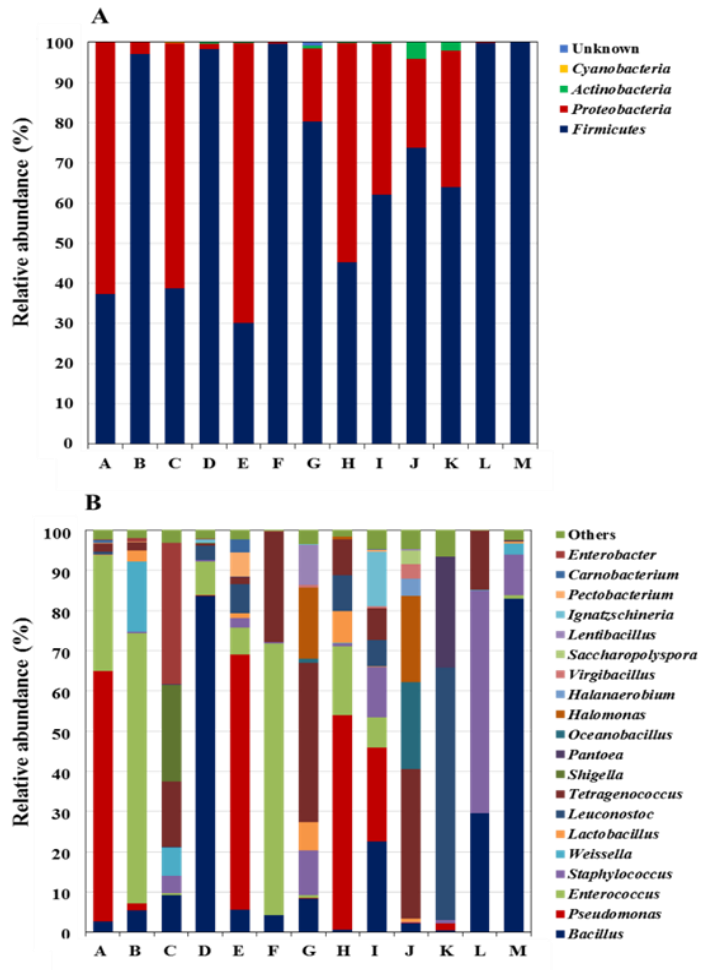

Figure II-1. Bacterial community changes in ten traditional- and three commercial doenjang. Data portray phylum (A) and genus (B) level analyses of bacterial 16S rRNA gene sequences derived from the doenjang samples. The 16s rRNA gene sequences with more than 300 bp were classified using the RDP naïve Bayesian rRNA Classifier at an 80% confidence threshold. The 'others' in panels B are composed of the genera, each showing a percentage of reads <1.0% of the total reads in all samples.

Figure II-2. Relative abundance of genus level taxa. Each column in the heat-map represents one doenjang sample, whilst each row represents a species level phylotype. The color intensity of the panel is proportional to the abundance of OTUs (max 84%).

II-3-3. Biogenic amine contents of doenjang samples

The level of nine types of biogenic amines were confirmed, because generally histamine, putrescine, cadaverine, tyramine, tryptamine, β-phenylethylamine, spermine, and spermidine are known that it is the most important biogenic amines occurring in foods. Figure II-3 shows different biogenic amine contents in 13 doenjang samples and the range of total biogenic amines were 55.74-4016.96 mg/kg in sample K and H, respectively. Tyramine (0-657.31 mg/kg), Put (26.96-821.35 mg/kg), Cad (9.15-47.45 mg/kg), SPD (0-42.83 mg/kg), SPM (0-5.25 mg/kg), and AGM (0-40.19 mg/kg) were detected. In the previous reports concerning fermented soy bean products, histamine, putrescine, cadaverine and tyramine were generally detected at the high concentration (Shukla, et al. 2014). Especially, histamine has been implicated as the causative agent in several outbreaks of food poisoning (Shalaby, 1996). The US Food and Drug Administration (FDA) have suggested histamine at a concentration of 500 mg/kg in fish to be hazardous for human health (Food and Drug Administration, 2001). On the other hand, Brink et al. (1990) reported that 100 - 800 mg/kg of tyramine and 30 mg/kg of phenylethylamine in foods are toxic. In this study, HIS (0-547.46 mg/kg), TRM (0-438.52 mg/kg), 2-PHA (4.07-2259.12 mg/kg) were detected in traditional doenjang samples. Three of

thirteen samples (sample C: 547.46 mg/kg, D: 525.75 mg/kg, and F: 524.mg/kg) for histamine exceeded the maximum limit (500 mg/kg) of FDA.

Several samples exceeded the amount recommend BAs concentration. Overall, the concentration of BAs in traditional doenjang was much higher than that in commercial doenjang, even spermine and histamine were not detected in three commercial doenjang (K, L, and M). In particular, in sample K, only putrescine (44.55 mg/kg), cadaverine (7.12 mg/kg), and 2-phenylalanine (4.07 mg/kg) were detected with low content. The main environment factors affecting microbial activities in foods are temperature, salt concentration, and pH. These factors can influence the formation of BAs in two ways. In first instance, they are responsible for the overall metabolism of the decarboxylating cells. In addition, the activity of decarboxylases depends on the same parameters. Another important factor producing BA is fermentation period. The process of commercial doenjang is performed in clean space and used Koji or starter instead of rice straw to control microorganism and doenjang quality. In addition, barley is used as carbohydrate source helping growth of starter. The using of starter can reduce the fermentation period by half and allow easy control of quality, consequentially, starter cultures are effective in reducing the amount of BA generated during the fermentation. Although

the presence of BAs in foods leads to a threat to human health, legislation establishing the threshold level about other BAs are not established in fermentation food yet.

PUT	CAD	TRM	2-PHA	SPD	SPM	HIM	TYM	AGM		
533.77 (71.28)	40.07 (5.18)	166.43 (22.84)	99.26 (18.45)	24.04 (3.15)	4.99 (0.81)	472.03 (92.96)	433.83 (132.88)	4.90 (1.00)	A	Traditional doenjang
561.08 (64.4)	20.97 (2.54)	19.45 (3.73)	377.55 (66.29)	5.60 (1.32)	1.69 (0.33)	196.22 (50.39)	345.02 (122.75)	39.30 (15.13)	B	
212.60 (10.31)	9.15 (1.52)	438.52 (77.85)	536.62 (99.55)	N.D.	N.D.	547.46 (104.38)	657.31 (213.26)	N.D.	C	
747.26 (125.85)	31.01 (2.35)	256.10 (34.42)	131.14 (17.16)	12.08 (1.79)	2.28 (0.12)	525.75 (89.62)	565.34 (233.3)	10.26 (4.63)	D	
691.96 (152.63)	44.21 (5.26)	134.29 (19.68)	589.94 (109.33)	10.70 (1.36)	4.33 (2.85)	280.43 (97.56)	440.79 (196.72)	40.19 (22.69)	E	
264.72 (16.77)	14.42 (1.71)	62.60 (5.47)	144.33 (11.26)	21.25 (1.66)	5.25 (0.77)	524.19 (51.21)	626.06 (202.5)	N.D.	F	
619.46 (103.35)	25.12 (2.67)	77.80 (10.36)	733.60 (86.09)	4.82 (0.47)	2.38 (0.4)	190.91 (51.21)	455.64 (205.02)	N.D.	G	
821.35 (247.98)	47.45 (11.96)	190.95 (65.93)	2259.12 (604.19)	24.83 (6.58)	3.13 (0.83)	126.97 (48.61)	543.17 (306.27)	N.D.	H	
368.83 (69.04)	20.65 (3.12)	17.30 (2.96)	50.85 (9.01)	6.62 0.49)	N.D.	189.69 (48.61)	34.40 (10.25)	N.D.	I	
294.69 (14.96)	26.33 (2.16)	9.36 (1.71)	337.13 (54.24)	13.41 (2.38)	4.15 (0.76)	33.14 (10.13)	255.16 (130.84)	N.D.	J	
44.55 (6.17)	7.12 (1.83)	N.D.	4.07 (2.38)	N.D.	N.D.	N.D.	N.D.	N.D.	K	Commercial doenjang
66.53 (2.13)	8.84 (1.00)	5.36 (0.29)	32.58 (2.47)	3.54 (0.55)	N.D.	N.D.	117.72 (14.93)	99.00 (11.61)	L	
26.96 (1.69)	3.95 (0.41)	42.22 (2.33)	6.89 (0.17)	9.24 (0.56)	N.D.	N.D.	N.D.	N.D.	M	

Figure II-3. Heat-map of biogenic amine content (mg/kg) in soy bean paste samples of Doenjang. Biogenic amines of 25g doenjang samples were extracted with 0.1N HCl 50 mL and centrifuged. An aliquot (2ml) of extract was mixed with 1ml of 2M NaOH and 10 ul benzoyl chloride in a test tube and incubated at 30°C for 40 min. after then, saturated NaCl (25%) 2ml was added to each tube and the content was extracted with 3 mL of diethylether and centrifuged. The upper layer (2mL) was transferred in to a tube and evaporated under stream of nitrogen. Abbreviation: PUT: putrescine, CAD: cadaverine, TRM: tryptamine, 2-PhA: 2-phenylalanine, SPD: spermidine, SPM: spermine, HIM: histamine, TYM: Tyramine, AGM: agmatine, ND: not detected

II-3-4. Structure of cultural bacterial communities isolated from traditional doenjang

The number of bacteria in each doenjang sample is presented in **Table II-5**. Although all doenjang products were made in the same year (2012-2013) and fermented approximately one year, the bacterial counts showed big difference among samples. The bacterial number was considerably influenced by the NaCl concentration of the plating medium and proportionally decreased by the addition of NaCl. Sample H has the greatest number of bacteria on TSA supplemented with 5% NaCl, but, the bacterial count decreased about 200 fold on 15% TSA. In sample J, the smallest bacteria were detected on all TSA added 5%, 10%, and 15%, even the number was less than that the number of bacteria counted on 15% TSA in other samples.

A total of 432 bacterial strains were isolated from six traditional doenjang based on showing high histamine concentration and low histamine concentration, and were identified as 7 genus and 30 species (Table II-6) on TSA media containing 5, 10, and 15% NaCl. Thirty species were consisted with sixteen species in the genus *Bacillus*, one species in the genus *Brevibacterium*, one species in the genus *Kocuria*, one species in the *Lentibacillus*, four species in the genus *Ocaeanobacillus*, three species in the genus *Staphylococcus*, two species in the genus *Tetragenococcus*, and two

species in the genus *Virgibacillus*. Among the genus *Bacillus, B. licheniformis* (119 isolates) were the predominant species, followed by *B. siamensis* (86 isolates), *B. subtilis* subsp. *subtilis*, (47 isolates), and *B. sonorensis* (36 isolates). Despite the genus *Bacillus* was predominated in all samples on TSA containing 5% and 10% NaCl, only three (*B. licheniformis, B. siamensis*, and *B. sonorensis*) of sixteen species in the *Bacillus* genus were detected on TSA added 15% NaCl. Previous studies and our result indicated that *B. subtilis* was the predominant organism, but this result was in disagreement with bacterial structure identified by culture-independent methods. As the NaCl concentration increased, the isolated bacterial population shifted from *Bacillus* spp. to halotolerant or halophilic bacteria including *Tetragenococcus, Oceanobacillus, Brevibacterium* and *Staphylococci.*

In the case of LAB, only one genera, *Tetragenococcus*, was identified. *Tetragenococcus halophilus* subsp. *flandriensis* and *Tetragenococcus halophilus* subsp. *halophilus* were detected in sample C, H, and I on only TSA containing 15% NaCl, but those two species of *Tetragenococcu*s were isolated on TSA containing 5% and 10% NaCl in sample J. The genus of *Weissella*, *Enterococcus*, and *Leuconostoc* were detected in doenjang samples analyzed by culture independent methods, however, those bacteria were not isolated by culture dependent method. The results

are assumed that media condition added salt is harsh for LAB to grow nevertheless the our culture conditions covered most of the major genera identified by both culture dependent- and culture independent- methods (Cho, et al. 2007, Kim, et al. 2009, Jeong, et al. 2014).

In many studies, the genus of *Staphylococcus* is the major species and very commonly isolated from meju, but its abundance showed a tendency to rapidly decrease after brining. These tendency is in agreement with our result that only 5 isolates were identified as the genus of *Staphylococcus*. The *Staphylococcus hominis* subsp. *hominis* was isolated from sample C, F, and J (1 isolates in each sample). *Staphylococcus nepalensis* (1 isolates) and *Staphylococcus epidemidis* (1 isolates) were isolated only in sample C. All the species of *Staphylococci* founded in this research were coagulase-negative staphylococci (CNS). CNS are widespread in nature and are part of the skin flora, support the hypothesis that the genus of *Staphylococcus* was derived from the human and the facility during the meju processing. In addition some CNS are even used as starter culture for dry fermented sausage and cheese to improve their aromatic and color. Therefore, I speculated that CNS founded in doenjang are probably not harmful for human.

Table II-5. Numbers of bacteria counted on TSA supplemented with 5, 10, and 15% NaCl

Sample	C	D	F	H	I	J
5%	1.8×10^7	4.1×10^7	1.4×10^8	5.7×10^8	9.0×10^7	8.8×10^5
10%	8.1×10^6	2.1×10^7	1.3×10^8	2.0×10^8	2.5×10^7	7.1×10^5
15%	2.9×10^6	1.6×10^6	9.5×10^7	2.8×10^6	1.2×10^6	1.6×10^5

[a]The NaCl concentration in TSA is 0.5% (w/v), and others indicate the final concentrations of NaCl in the media. Cell counts were repeated three times independently.

Table II-6. Number of isolates from traditional doenjang samples summarized at the species level

Species	C			D			F			H			I			J			Total
	5%	10%	15%	5%	10%	15%	5%	10%	15%	5%	10%	15%	5%	10%	15%	5%	10%	15%	
Bacillus aerius																		1	1
Bacillus aerophilus	1															1	1		3
Bacillus altitudinis																1			1
Bacillus amyloliquefaciens subsp. *plantarum*	2	4															1		7
Bacillus atrophaeus				3	6												1		10
Bacillus clausii																2	2		4
Bacillus licheniformis	1	1	4	7	2	16	10	14	16	2	2	3	3	3	4	13	10	8	119
Bacillus methylotrophicus				3							2		3	3					11
Bacillus pumilus													2	2		2	1		7
Bacillus safensis	1	2																	3
Bacillus siamensis	15	13		7	8		1	1	1	10	2		14	13		1			86
Bacillus sonorensis			1	2	1	8	2	2		1	1	9			6		2	1	36
Bacillus sp.																	1		1
Bacillus subtilis subsp. *inaquosorum*	2	2		1							1			1		2	1		10
Bacillus subtilis subsp. *subtilis*				3	8		9	6		9	7		1	3		1			47
Bacillus tequilensis		2					1	2			6						1		12
Brevibacterium halotolerans	3	1																	4
Kocuria marina							1												1
Lentibacillus salicampi																	2	7	9

Oceanobacillus caeni										1									1
Oceanobacillus oncorhynchi subsp. *incaldanensis*												4			2			1	7
Oceanobacillus oncorhynchi subsp. *oncorhynchi*											2	3							5
Oceanobacillus sojae									1		1	4							6
Staphylococcus epidemidis			1																1
Staphylococcus hominis subsp. *hominis*			1								1						1		3
Staphylococcus nepalensis			1																1
Tetragenococcus halophilus subsp. *flandriensis*			11												4	2	1	4	22
Tetragenococcus halophilus subsp. *halophilus*			2									1			7		1		11
Virgibacillus campisalis												1							1
Virgibacillus dokdonensis																	1	1	2
Total	25	25	21	26	25	24	24	25	18	23	25	25	23	25	23	25	27	23	432

II-3-5. Evaluation of protease, amylase, and lipase activities

Amylase, protease, and lipase activities of the isolates were determined by the degradation of substrates supplemented in the medium to investigate the role of represented species in doenjang. The enzyme activities were tested on TSA media supplemented with 5% NaCl. Generally, the activities of isolates within a species were notably similar (**Table II-7)**. Only 1.68% of *B. licheniformis* showed protease activity, whereas all strains had the protease activity on TSA media (data not shown), as mentioned previous studies, that sodium chloride has a strong inhibitory effect on protease activity. However, 100% of *B. siamensis* and *B. subtilis* subsp. *subtilis* showed strong protease activity on TSA added 5% NaCl. Interestingly, almost isolates regardless of genus could hydrolyze tributyrin. 99.2% of *B. licheniformis* and 95.7% *B. subtilis* subsp. *subtilis* and all other species showed amylase activities. It seems that the lipase activity is not affected by addition of sodium chloride. Among 13 species, only two species (*B. siamensis*: 94.2% and *B. subtilis* subsp. *subtilis*: 91.5%) showed lipase activity. Although, in vitro test, *B. siamensis* was the most suitable species for doenjang starter showing excellent protease, lipase, and amylase activities, this species is not registered for the food additive list of Ministry of Food and Drug Safety (MFDS). Thus, I selected 12 strains belonging to *B. subtilis*,

authorized species, based on enzyme activities and hemolytic activity, and then used the 12 strains for further studies to select doenjang starter.

Table II-7. Amylase, protease, lipase, and hemolysis activities of the represented species on TSA media supplemented with 5% NaCl

Species	TSA supplemented 5% with NaCl				
	Isolates No.	protease	lipase	amylase	hemolysin
B. licheniformis	119	2 (1.68%)	118 (99.2%)	0	71 (59.7%)
Bacillus siamensis	86	86 (100%)	86 (100%)	81 (94.2%)	37 (43.0%)
Bacillus subtilis subsp. *subtilis*	47	47 (100%)	45 (95.7%)	43 (91.5%)	5 (10.6%)
Oceanobacillus oncorhynchi subsp. *incaldanensis*	7	0	7 (100%)	0	0
Oceanobacillus oncorhynchi subsp. *oncorhynchi*	5	0	5 (100%)	0	0
Oceanobacillus sojae	6	0	6 (100%)	0	0
Staphylococcus epidemidis	1	0	1 (100%)	0	0
Staphylococcus hominis subsp. *hominis*	3	0	3 (100%)	0	0
Staphylococcus nepalensis	1	0	1 (100%)	0	0
Tetragenococcus halophilus subsp. *flandriensis*	22	0	0	0	0
Tetragenococcus halophilus subsp. *halophilus*	11	0	0	0	0
Virgibacillus campisalis	1	1 (100%)	1 (100%)	0	0
Virgibacillus dokdonensis	2	2 (100%)	2 (100%)	0	0

II-3-6. Evaluation of essential and toxic elements of doenjang

Essential and toxic elements of six doenjang samples were estimated by inductively coupled plasma mass spectrometry (ICP-MS). According to WHO, Zn, Se, Cu and Cr are the essential elements, while Mn is probable essential elements (WHO, 1996). The World Health Organization (FAO) (WHO, 1982, WHO, 1996, FDA, 2001) has reported five types of critical levels for minor and trace elements: provisional maximum tolerable daily intake (PMTDI) level, provisional tolerable weekly intake level (PTWI), dietary reference intakes (DRI), total dietary intakes (TDI) and an upper level (UL). According to these values, the DRI (mg/kg) for Cr, Cu, Zn, and Se are 0.015-0.030, 0.7-0.9, 8-11, and 0.05-2.0 mg/kg, respectively.

In this study, the results of minor elements were Cr (0-0.648 mg/kg), Cu (0.919-3.012 mg/kg), Mn (12.21-17.51 mg/kg), and Zn (17.26-24.27 mg/kg) (Table II-9). All were much higher than critical level proposed by FAO. The specified UL for Cu is 3-10 mg/kg, while PMTDI for Cu and Zn are 0.05-0.5 mg/kg and 1.0 mg/kg, respectively. The result obtained for nutritional elements Cu (0.919-3.012 mg/kg) was within these specified limit based on the UL, whereas the level of Cu and Zn (17.26-24.27 mg/kg) were exceed more than 6.2- and 24.3- times.

Cr is essential nutrient, which play a role in the metabolism of glucose and protein and commonly founded in food. However, ingestion of Cr cause detrimental physiological effects such as stomach ulcer and anemia in blood as well as cancer (Bremner, 1998). According to Korea Food and Drug Administration (KFDA, Risk profile of Chromium), the total daily amount of Cr intake from typical Korean foods, was 0.089 mg/kg (person/day). In this study, in sample H (0.051 mg/kg), I (0.196 mg/kg), and J (0.648 mg/kg), Cr was detected. Considered that total Cr levels in most foods typically range from < 10 to 1,300 μg/kg, the amount of Cr detected in doenjang samples (I and J) was comparatively high. However, Lee et al. insisted the difficulty to analyze Cr in complex food matrices by using ICP-MS because the major isotope (52Cr) is masked by residual carbon or high chloride concentrations in the sample matrix and the plasma gas (Lee, et al. 2013). Therefore, I cannot assert that whether consumption of traditional doenjang containing high level of Cr is safe or not, so, further investigation is required to confirm the Cr level and safety of doenjang.

Trace elements including Ca, Co, Fe, K, Mg, and Na were confirmed (Table II-8). Toxic trace elements including As, Cd, and Pb were measured, however none of these three toxic trace elements were detected in all doenjang samples.

Table II-8. Analytical results for doenjang samples (mg/kg) obtained by ICP

Elements	Sample					
	Concentration (mg/kg)					
	C	**D**	**F**	**H**	**I**	**J**
Minor elements						
Cr	0.000	0.000	0.000	0.051	0.196	0.648
Cu	2.625	1.948	1.018	1.433	0.919	3.012
Mn	13.16	17.26	13.22	12.21	13.20	17.51
Zn	18.31	21.62	19.66	17.26	18.01	24.27
Se	0.000	0.000	0.000	0.000	0.000	0.000
Trace elements						
Ca	889.7	1394	931.2	1053	1276	1166
Co	0.000	0.000	0.000	0.000	0.142	0.120
Fe	30.22	43.40	30.00	32.98	41.55	38.46
K	11730	12180	9718	8584	8167	9186
Mg	955.5	2307	1252	1804	2760	1943
Na	34600	39900	40280	34900	40640	34420
Toxic trace						
As	0.000	0.000	0.000	0.000	0.000	0.000
Cd	0.000	0.000	0.000	0.000	0.000	0.000
Pb	0.000	0.000	0.000	0.000	0.000	0.000

II-3-7. Quantitative assay of the enzyme activities of *B. subtilis* strains

Fibrinolytic activity is one of important fuctional properties in doenjang and various fibrinolytic enzymes such as streptokinase and nattokinase can be produced by microorganisms (Peng, et al. 2003). Threrefore, fibrinolytic enzyme activities of 47 *B. subtilis* strains were assayed in crude enzyme extracts from each strains for screening doenjang starter candidates and the acticities were quantified in comparison to a plasmin standard curve. Most of the strains, especially strains 5TDH25 (6 unit/ml), 5TDI8 (6 unit/ml), and 10TDI13 (6 unit/ml), showed strong fibrinolytic enzyme ativities. Among them, 12 strains showing the highest fibrinolytic activities were selected (Table II-9, Figure II-4) for further study. Amylase, protease, and lipase are very impotant enzymes to degrade the macro-molecules of soybean and those enzymes produced by various microorganisms are essential for doenjang fermentation. Thus, the amylase, protease, and lipase activities of 12 selected *B. subtilis* strains were evaluated by cultivating the strains on TSA containing 5% NaCl considering high salt concentration of doenjang. The selected strains were exhibited strong amylase, protease and lipase activities (Table II-9), which inferred that the selected strains can produce those enzymes and they could have the avtivities in doenjang.

Table II-9. Quantitative assays of amylase, protease, lipase, and fibrinolytic activities of 12 selected *Bacillus* strains

Strain	TSA (5% NaCl)			Fibrinolytic activity (unit)[b]
	Amylase[a]	Proteolytic[a]	Lipase[a]	
5TDH4	++++	+++	+++	4.5
5TDH11	++++	+++	+++	5
5TDH12	++++	++	+++	5.45
5TDH25	++++	++	+++	6
10TDH11	++++	++++	+++	5
10TDH18	++++	++	+++	5
5TDI8	++++	+++	+++	6
10TDI3	++++	++++	++	5.5
10TDI13	++++	++++	++++	6
5TDJ16	++++	+++	++++	3
5TDF11	++++	+++	++++	5
15TDI8	++++	++++	+++	5.5

[a]Diameter (mm) of halo zones around disk: -, negative; 0.01-1.5; ++, 1.5-3.0; +++, 3.0-4.5; ++++,>4.5

Figure II-4. Fibrinolytic activity of cultural supernatant from bacterial strains isolated from doenjang. Plates were photographed after incubation for 18 hours at 30℃.

II-3-8. Detection of enterotoxin genes and cytotoxicity activity

PCR was used to test chromosomal DNA from the 12 *Bacillus* strains for the presence of enterotoxin genes. *Bacillus cereus* ATCC 14579 was used as positive strain for the HBL and NHE enterotoxins. Previous study showed that almost all strains of *B. cereus* and *Bacillus thuringiensis* from the food-poisoning and food-borne ecosystems carried hemolysin BL (HBL) non-hemolytic enterotoxin (NHE) genes, and the *bceT* gene, which encodes the single-component toxin enterotoxin T, was also widely distributed (57 and 71% among food-poisoning and food-borne strains, respectively) (Hansen, et al. 2001). In addition, several studies reported that *Bacillus* sp. such as *B. licheniformis*, *B. subtilis*, and *B. pumilus* have rarely been linked to incidents of food-borne illness as produce active *B. cereus* toxins (Drobniewski, et al. 1993, Griffiths, et al. 1990). Nevertheless, none of 12 starter candidates isolated form doenjang samples carried enterotoxin genes, while ATCC14579 had seven enterotoxin gene (*nheA, nheB, nheC, hblA, hblC, bceT,* and *cytK*) (Table II-10). In addition, INT-407 cells incubated with each strain did not show increased lysis above the baseline, indicating that the 12 *B. subtilis* strains were not cytotoxic (data not shown)

Table II-10. Distribution of enterotoxin genes in starter candidates isolated from doenjang and *B. cereus* reference strain ATCC 14579

Strain	*nheA*	*nheB*	*nheC*	*hblA*	*hblC*	*bceT*	*cytK*
ATCC 14579	+	+	+	+	+	+	+
5TDH4	-	-	-	-	-	-	-
5TDH11	-	-	-	-	-	-	-
5TDH12	-	-	-	-	-	-	-
5TDH25	-	-	-	-	-	-	-
10TDH11	-	-	-	-	-	-	-
10TDH18	-	-	-	-	-	-	-
5TDI8	-	-	-	-	-	-	-
10TDI3	-	-	-	-	-	-	-
10TDI13	-	-	-	-	-	-	-
5TDJ16	-	-	-	-	-	-	-
5TDF11	-	-	-	-	-	-	-
15TDI8	-	-	-	-	-	-	-

[a]+, a PCR product of the expected size was observed; -, no PCR product was observed.

II-3-9. Antibiotic susceptibility

Antibiotic resistance can cause significant danger and the fermented food can be considered as the main route of transmission of antibiotic resistant bacteria between the animal and human population, therefore, antibiotic resistance is one of the most important safety issues for starter selection. Table II-11 shows the antibiotic resistance to sixteen types of antibiotics for the 12 *B. subtilis*, which are starter candidates showed high enzyme activities. The antibiotics were selected from the antibiotic list provided by European Food Safety Authority for *Bacillus* spp. (EFSA, 2012) because the guidelines have not yet been developed to determine the antibiotic susceptibility of microorganisms in Korea. Among 16 antibiotics, all 12 strains were against Ampicillin, Ciprofloxacin, Gentamycin, Kanamycin, Linezolid, Neomycin, Penicillin G, Rifampicin, Tetracycline, and Vancomycin, which suggested species specific intrinsic resistance, whereas they exhibited different antibiotic resistance to the rest antibiotics, which suggested strains specific resistance rather than species specific. All bacteria strains were susceptible toward Chloramphenicol, Erythromycin, Lincomycin, Oxacillin, and Streptomycin. Interestingly, 5 strain of *B. subtilis* were exhibited antibiotic resistance to Sulphamethoxazolec. *B. subtilis* strain-specific resistance to Sulphamethoxazolec assumed that this was in fact

acquired resistance. Taken together, our results indicate that most *Bacillus* strains tested in this study were sensitive to the antibiotics provided by EFSA (2012), therefore the strains, which were susceptible to 16 antibiotics, are considered as safe for human consumption. However, the further study is required that starter candidates should be confirmed the presence of acquired genes for antimicrobial resistance, because the acquired genes can be spread to humans through food consumption.

Table II-11. Antibiotic resistance of *Bacillus* strains using disk methods

Strains	Antibiotics disc (μg)[a]															
	AMP	**CHL**	**CIP**	**ERY**	**GEN**	**KAN**	**LIN**	**LZD**	**N**	**OXA**	**PEN**	**RIF**	**STR**	**TMP/SMX[c]**	**TET**	**VAN**
	10	**30**	**5**	**15**	**10**	**30**	**15**	**10**	**30**	**1**	**10**	**10**	**10**	**25**	**30**	**30**
14579	R[b]	S	S	S	S	S	S	S	S	R	R	S	S	S	S	S
5TDH4	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S
5TDH11	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S
5TDH12	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S
5TDH25	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
10TDH11	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
10TDH18	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5TDI8	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S
10TDI3	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
10TDI13	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5TDJ16	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5TDF11	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
15TDI8	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S

[a]Antibiotic-impregnated discs (6 mm) with amount, μg shown in brackets

[b] Diameter of inhibition from three individual experiments. (S): Sensible; (R): resistant

[c] Trimethoprim/Sulphamethoxazole (TMP/SMX) 19:1 The abbreviation represent antibiotics as follows: AMP, Ampicillin; CHL, Chloramphenicol; CIP Ciprofloxacin; ERY, Erythromycin; GEN, Gentamycin; KAN, Kanamycin, Lin, Lincomycin; LZD, Linezolid, N, Neomycin; OXA, Oxacillin; PEN, Penicillin G; RIF, Rifampicin; STR, Streptomycin; TMP/SMX, Trimethoprim/Sulphamethoxazole; TET, Tetracyclin; VAN, Vancomycin.

II-3-10. Quantitative evaluation of the anti-pathogenic activities

The anti-pathogenic activities of the 12 *B. subtilis* strains were quantitatively evaluated. Although they were the same species, inhibitory spectra were different depending on strains. Most of the strain have anti-pathogenic activities against four pathogens (*B. cereus* ATCC27348, *E. coli* O157:H7, *S. aureus* KCTC3881, and *L. monocytogenes* KCTC13064). All strains showed relatively strong inhibitory activities against *E. coli* and *L. monocytogenes* but, showed relatively weak activities against *B. cereus* and *S. aureus*. Interestingly, most of *B. subtilis* showed inhibitory activity against to *B. cereus*, even they belong to the same genus. Strain 5TDI8 and 15TDI8 showed inhibitory acticity against *S. aureus*, but showed no antibacterial activity against *B. cereus*, which is consistent with previous reported results (Jeon, *et al.* 2016). These results suggested that each strain may produce different kinds of antibacterial compounds.

Table II-12. Quantitative assays of fibrinolytic activities of 13 selected *Bacillus* strains

Strain	Anti-pathogenic activity			
	B. cereus **ATCC27348a**	***E. coli*** **O157:H7a**	***S. aureus*** **KCTC3881a**	***L. monocytogenes*** **KCTC13064b**
10TDD5	+	+++	++	++++
5TDH4	+	+++	++	+++++
5TDH11	+	+++	-	+++++
5TDH12	+	+++	-	+++++
5TDH25	+	+++	++	+++++
10TDH11	+	+++	++	+++++
10TDH18	+	++	+	+++++
5TDI8	-	++++	++	++++
10TDI3	+	++++	+	+++++
10TDI13	+	++++	+	+++++
5TDJ16	+	++	++	+++
5TDF11	+	+++	+	++
15TDI8	-	++	+	++

[a]Diameter (mm) of inhibition zones around colonies showing antimicrobial activities: –, negative; +, 0.01–1.0; ++, 1.0–2.0; +++, 2.0–3.0; ++++, 3.0–4.0; +++++, >4.0.

[b]Diameter (mm) of inhibition zones around colonies showing antimicrobial activities: –, negative; +, 0.01–2.0; ++, 2.0–4.0; +++, 4.0–6.0; ++++, 6.0–8.0; +++++, >8.0.

II-3-11. Biogenic amine production by 12 *B. subtilis* strains

Cadaverine, putrescine, tyramine, and histamine are prevailingly detected in fermented food (Alvarez, et al. 2014) and those biogenic amines are produced by the decarboxylation of ornithine, lysine, tyrosine, and histidine, respectively. The biogenic amine concentration produced by 12 *B. subtilis* strains was evaluated in TSB media supplinented with 0.2% amine precursor (ornithine, tyrosine, histidine, and lysine). Twelve *B. subtilis* strains produced very small amount of biogenic amines. The levels of cadaverine, histamine, putrescine, and tyramine were only 0.3 ppm, 0.52 ppm, 0.6 ppm, and 0.22 ppm, respectively (Figure II-5). The variety of microorganisms including *Pseudomonas* spp. *Escherichia coli*, *Klebsiella pneumonia*, and *Entercococcus* can produce biogenic amines and they have biogenic amine decarboxylase genes in their genome. In addition, the ability to produce biogenic amines is considered as a strains-specific characteristic and that strain can acquire the BA producing capacity by horizontal gene transfer (Ladero, et al. 2012). However, information regarding the biogenic amine decarboxylase activity of *Bacillus* strains isolated from food is insufficient and most studies were focused on the biogenic amine production by species of the family *Enterobacteriaceae* and *Staphylococcaceae* from different origin such as sausage, meat, and fish (Bermúdez,

et al. 2012, Veciana-Nogués et al. 2004, Chang et al. 2012, Landeta G et al. 2007). In general, the average level of putrescine and cadaverine produced by the spicies belonging to *Bacillus* (0-5 mg/L) is relatively much lower than that produced by *Staphylococcus*, ranged from 25 to 1000 mg/L (Martín, et al. 2006), suggersting that the use of *Bacillus* strains as doenjang starter could reduce the biogenic amine contents.

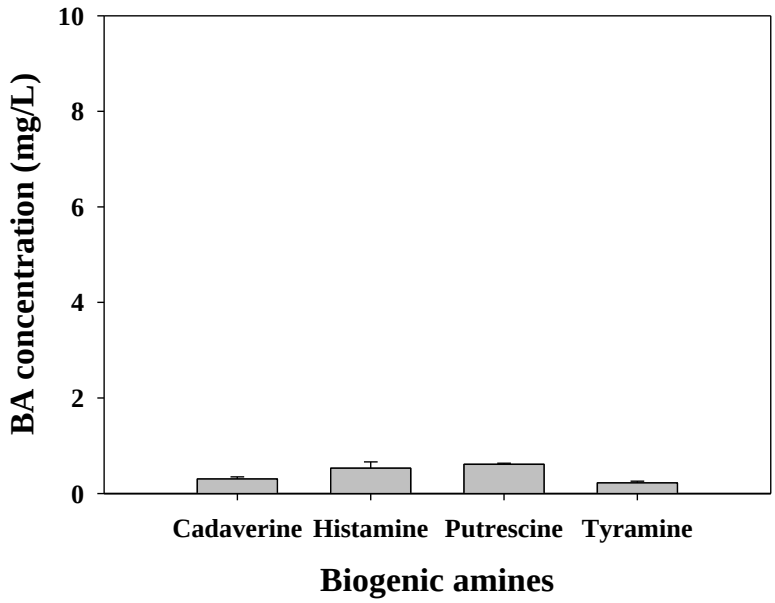

Figure II-5. Biogenic amine concentration produced by 12 selected *B. subtilis* strains in medium supplemented with precursors

II-4. Conclusions

In this study, bacterial communities and biogenic amine contents in ten traditional and three commercial doenjang products were surveyed. Analysis of bacterial community using pyrosequencing and the level of biogenic amines showed that they were different depending on the sample. Overall, the levels of biogenic amines in traditional doenjang were much higher than those in commercial doenjang. However, it was unable to confirm the correlation between microbial communities and biogenic amine contents, which is supposed that varied microorganism are involved in doenjang fermentation and their ratio changes depending on the fermentation period. Therefore, it is necessary to conduct an experiment by the period of fermentation to clarify the correlation between microbial communities and biogenic amine content. To select a doenjang starter, the features and safety of 12 *B. subtilis* strains screened by fibrinolytic activity was investigated. *B. subtilis* 10TDI13 was selected as a doenjang starter based on the enzyme activities, antibiotic resistance, and biogenic amine production, suggesting that the selected strain satisfied all requirement that can be used as appropriate starter candidates to produce standardized doenjang with high quality and safety. In the next step, therefore,

further studies of doenjang prepared with strain 10TDI13 is necessary to prove the rationales for selection of starter culture.

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Chapter III.

Comprehensive phylogeny and metabolic functions of *Bacillus subtilis* related strains using pan-genome analysis

III-1. Introduction

The species of *Bacillus subtilis*, a Gram-positive, spore-forming, fermentative, aerobic, rod-shaped and catalase-positive bacterium, is one of the most important species in science area and food industry. The *B. subtilis* species complex is a tight assemblage of closely related species including *Bacillus amyloliquefaciens* (Priest, et al. 1987), *Bacillus atrophaeus* (Nakamura, 1989), *Bacillus axarquiensis*, *Bacillus malacitensis* (Ruiz-Garcı´a, et al. 2005b), *Bacillus mojavensis* (Roberts, et al. 1994), *Bacillus sonorensis* (Palmisano, et al. 2001), *Bacillus tequilensis* (Gatson, et al. 2006), *Bacillus vallismortis* (Roberts, al. 1996) and *Bacillus velezensis* (Ruiz-Garcı´a, et al. 2005a). Although, DNA–DNA hybridization values of those species show below 70 %, they cannot be differentiated on the basis of 16S rRNA. In particular, *B. subtilis* consisting of three subspecies, *B. subtilis* subsp. *subtilis*, *B. subtilis* subsp. *inaquosorum*, and *B. subtilis* subsp. *spizizenii* is phenotypically extremely similar and they are not clearly differentiated on the basis of their phenotypic properties and 16S rRNA gene sequences (Roberts, et al. 1994, Nakamura, et al. 1999). In general, the DDH value, the gold standard of the prokaryotic species delineation (Goris, et al. 2007), is used to distinguished species.

However, the previous studies proved that DDH value cannot clearly distinct three subspecies groups. (Nakamura, et al. 1999, Rooney, et al. 2009). Therefore, re-evaluation of the taxonomic status using a genome-based method is necessary to reclassify the *B. subtilis* genomes at the subspecies level.

To obtain insight into the genetic characteristics of *B. subtilis* genome, I analyzed the taxonomic status and molecular phenotypes using the whole genome sequences of 99 *B. subtilis* strains including strain 10TDI13, which was isolated from Korean traditional doenjang, available in GenBank.

III-2. Materials and Methods

III-2-1. Bacterial strains and DNA isolation

B. subtilis 10TDI13 was isolated from Korean traditional Doenjang. *B. subtilis* 10TDI13 was cultured in TSA broth (DIFCO, USA) at 30℃ for 24 h. Genomic DNA was isolated by the method using phenol-chloroform as described previously (Neumann, et al. 1992).

III-2-2. Whole genome sequencing, de novo assembly and annotation

Whole-genome sequencing of *B. subtilis* 10TDI13 was conducted at the ChunLab Inc. (Seoul, South Korea) using the combination of an Illumina MiSeq platform (Illumina, San Diego, CA) and a PacBio RS II platform (Pacific Biosciences, Menlo Park, CA). A library was constructed using the PacBio library kit and the size was targeted to 10 kb. The generated sequencing reads were de novo assembled using the RS Hierarchical Genome Assembly Process (HGAP) protocol version 3.0 in SMRT Analysis version 2.3.0 (Pacific Biosciences, USA) or Canu assembler version 1.1.The genome sequences of the *B. subtilis* D12-5 was annotated by the National Center for Biotechnology Information (NCBI) Prokaryotic Genome Annotation

Pipeline (http://www.ncbi.nlm.nih.gov/genome/annotationprok/). Genome information of each strain for pan-core genome analysis is reported in Table III-1.

Table III-1. General features of *B. subtilis* strains

Name of strains or subspecies in Genbank	Genome status[a] (No. contigs)	Size (Mb)	GC content (%)	Total number of genes	Total number of protein coding sequences	Accession No.
2014-3557	D (76)	4.18	43.4	4402	4282	JYFL00000000.1
ATCC 13952	C (1)	3.88	45.8	3917	3793	CP009748
ATCC 19217	C (1)	3.96	46.4	3759	3630	CP009749
B-1	C (1)	3.94	46.5	3783	3630	CP009684
B4068	D (101)	3.96	43.4	4195	3943	JXHK00000000.1
B4069	D (68)	4.02	43.4	4267	4046	JXHL00000000.1
B4070	D (175)	4.20	43.4	4494	4244	JXHM00000000.1
B4071	D (74)	4.13	43.2	4399	4160	JXHN00000000.1
B4072	D (72)	4.02	43.4	4273	4045	JXHO00000000.1
B4073	D (100)	4.05	43.3	4304	4042	JXHP00000000.1
B4143	D (17)	4.10	43.7	4248	4109	JXLQ00000000.1
B4145	D (152)	4.32	43.3	4650	4360	JXHQ00000000
B4146	D (77)	4.18	43.3	4452	4252	JXHR00000000.1
B4417	D (49)	4.19	43.4	4403	4313	LJSM00000000.1
BEST7003	C (1)	4.04	43.9	4024	3885	AP012496
BS49	C (1)	4.25	43.5	4253	4125	LN649259
Bs-916	C (1)	3.98	46.5	3837	3689	CP009611
BSn5	C (1)	4.09	43.8	4240	4059	CP002468
BST	D (1)	4.04	43.9	4149	3955	JMNA00000000
C3	D (226)	4.18	43.9	4323	3313	JYOG00000000.1
D7XPN1	D (28)	4.08	43.8	4004	3033	JHCA00000000.1
E1	D (16)	4.11	43.6	4230	4113	CAUC00000000.1
E72	D (75)	4.17	43.4	4381	4296	JNCN00000000.1
GB03	D (77)	3.85	46.6	3702	3604	AYTJ00000000
gtP20b	D (88)	4.21	44.0	4209	4049	AEHM00000000
GXA-28	D (17)	4.26	43.6	4488	4343	JPNZ00000000.1

Hal1	D (745)	3.98	43.8	3931	3777	AMCA00000000
HJ5	C (1)	4.01	43.8	3954	3828	CP007173
HM-66	D (14)	4.36	42.9	4695	4251	JXBC00000000.1
10TDI13	C(2)	4.19	43.3	4446	4128	CP015222
HUK15	D (92)	4.25	43.5	4376	4201	LSMU00000000.1
JRS2	D (114)	4.06	43.6	4055	4045	CYHJ00000000.1
JRS6	D (80)	3.99	43.7	3965	3955	CYHN00000000.1
JRS7	D (211)	4.12	43.8	4024	4016	CYHO00000000.1
JRS9	D (125)	4.04	43.7	4021	4015	CYHS00000000.1
KATMIRA1933	D (125)	4.26	43.4	4500	4047	JMEF00000000.1
KCTC 1028	C (1)	4.22	43.5	4222	4094	CP11115
LK22	D (76)	4.10	43.7	4122	3999	LDUW00000000.1
MB418	D (288)	4.12	43.6	4339	4055	LYDW00000000.1
MB73/2	D (75)	4.17	43.4	4167	4082	AOTY00000000
Miyagi-4	D (407)	4.08	43.3	4398	4058	BALZ00000000.1
MS1577	D (24)	4.26	43.3	4507	4400	LFOC00000000.1
ND23	D (112)	4.18	43.9	4324	4089	LZCF00000000.1
NKYL29	D (22)	3.95	46.3	3787	3682	JPYY00000000.1
PS832	C (1)	4.22	43.5	4221	4090	CP010053
PTS-394	D (74)	4.01	43.7	3915	3847	AWXG00000000
PY79	C (1)	4.03	43.8	4010	3885	CP006881
QB928	C (1)	4.15	43.6	4344	4203	CP003783
QH-1	D (1)	4.03	43.7	4011	3914	AZQS00000000
S1-4	D (104)	4.45	43.1	4815	4538	ANIP01000001
SG6	C (1)	4.08	43.8	4053	3914	CP009796
SRCM101280	D (22)	4.13	43.5	4208	4128	LYUI00000000.1
subsp. *globigii* ATCC 49760	C (1)	4.18	43.2	4100	3950	CP014840
subsp. *inaquosorum* DE111	C (1)	4.14	43.9	4241	4066	CP013984
subsp. *inaquosorum* KCTC 13429	D (24)	4.34	43.7	4344	4185	AMXN00000000
subsp. *natto* BEST195	C (2)	4.11	43.5	4377	4083	AP011541
subsp. *natto* CGMCC 2108	C (7)	4.19	43.4	4488	4183	CP014471
subsp. niger PCI 246	D (2)	4.15	43.2	4073	3937	JMTJ00000000
subsp. *spizizenii* ATCC 6633	D (77)	3.98	43.8	4075	3925	ADGS00000000.1

subsp. *spizizenii* DV1-B-1	D (20)	3.97	43.6	4126	3833	AFSG00000000
subsp. *spizizenii* NRS 231	C (1)	4.03	43.9	4126	3966	CP010434
subsp. *spizizenii* RFWG1A3	D (25)	4.06	43.7	4171	3928	AJHL00000000.1
subsp. *spizizenii* RFWG1A4	D (17)	4.00	43.8	4101	3904	AJHM00000000.1
subsp. *spizizenii* RFWG4C10	D (16)	3.98	43.8	4069	3917	AJHN00000000.1
subsp. *spizizenii* RFWG5B15	D (15)	4.01	43.7	4116	3938	AJHO00000000.1
subsp. *spizizenii* str. W23	C (1)	4.03	43.9	4129	3963	CP002183
subsp. *spizizenii* TU-B-10	C (1)	4.21	43.8	4301	4086	CP002905
subsp. *subtilis* 3NA	C (1)	4.20	43.6	4199	4070	CP010314
subsp. *subtilis* 6051-HGW	C (1)	4.22	43.5	4223	4093	CP003329
subsp. *subtilis* ALBA01	D (28)	4.12	43.7	4287	4116	LVYH00000000.1
subsp. *subtilis* B4067	D (168)	4.31	43.3	4655	4357	JSXS00000000.1
subsp. *subtilis* BSD-2	C (1)	4.03	43.9	4133	3944	CP013654
subsp. *subtilis* CU1050	C (1)	4.06	43.9	4209	4064	CP014166
subsp. *subtilis* D12-5	C (1)	4.14	43.6	4336	3748	CP014858
subsp. *subtilis* delta6	C (1)	3.88	43.9	4061	3928	CP015975
subsp. *subtilis* NDfood	D (12)	4.06	43.7	4226	4137	JPVX00000000.1
subsp. *subtilis* NDmed	D (10)	4.06	43.7	4220	4135	JPVW00000000.1
subsp. *subtilis* str. 168	C (1)	4.22	43.5	4421	4175	NC_000964
subsp. *subtilis* str. AG1839	C (1)	4.19	43.5	4423	4286	CP008698
subsp. *subtilis* str. AUSI98	D (127)	4.35	43.5	4615	4399	AFSF00000000
subsp. *subtilis* str. BAB-1	C (1)	4.02	43.9	3977	3818	CP004405
subsp. *subtilis* str. BSP1	C (1)	4.04	43.9	4156	3854	CP003695
subsp. *subtilis* str. JH642	C (1)	4.19	43.5	4416	4235	CM000489
subsp. *subtilis* str. JH642 substr.	C (1)	4.19	43.5	4420	4285	CP007800
subsp. *subtilis* str. MP11	D (647)	3.93	43.7	4091	3608	APMX00000000.1
subsp. *subtilis* str. MP9	D (727)	3.95	43.8	3978	3732	APMW00000000.1
subsp. *subtilis* str. NCIB 3610	D (80)	4.29	43.3	4512	4306	CM000488.1
subsp. *subtilis* str. OH 131.1	C (1)	4.04	43.8	4171	4000	CP007409
subsp. *subtilis* str. RO-NN1	C (1)	4.01	43.9	3965	3802	CP002906
subsp. *subtilis* str. SC-8	D (17)	4.14	43.5	4329	4232	AGFW00000000
subsp. *subtilis* str. SMY	C (1)	4.21	43.5	4434	4242	CM000490
subsp. *subtilis* Y3	D (20)	4.04	43.9	4131	3986	LRFK00000000.1

SZMC 6179J	C (1)	4.20	43.6	4410	4276	CP015004
T30	C (1)	4.03	43.9	4129	3892	CP011051
TO-A	C (1)	4.06	43.8	4218	4094	CP005997
TO-A JPC	C (1)	4.09	43.8	4070	3927	CP011882
UD1022	C (1)	4.03	43.9	3970	3807	CP011534
WAUSV36	D (2)	4.24	43.4	4449	4110	LWLQ00000000.1
XF-1	C (1)	4.06	43.9	4023	3855	CP004019

III-2-3. Collection of genomic data and quality assessment

All genomes analyzed were downloaded from GenBank at the National Center for Biotechnology Information (NCBI- http://www.ncbi.nlm.nih.gov/) on the 18th of June 2016 to investigate the comprehensive characteristics of *B. subtilis*. All draft and complete genomes were downloaded; and then a few of genomes were excluded due to content and quality. The completeness and contamination of the genomic data were assessed by CheckM (Version 1.0.4) that is an automated method for evaluate the quality of genomes using lineage-specific marker genes (Parks, et al. 2015).

III-2-4. Relatedness analysis based on average nucleotide identity (ANI) and *in silico* DNA-DNA hybridization values

Whole genome sequences of strain 10TDI13, three type strains of *B. subtilis* subspecies, and their close relatives available in GenBank at the time of writing (August 2016) were used to investigate their relatedness based on average nucleotide identity (ANI) and *in silico* DNA-DNA hybridization (DDH) and their general features were described in Table III-1.

The pair-wise ANI values with the whole genomes were calculated using a stand-alone software, orthologous Average nucleotide identity Tool (OAT), available at

http://www.ezbiocloud.net/sw/oat (Lee, et al. 2015), with the following recommended parameters (minimum length, 700 bp; minimum identity, 70 %; minimum alignment, 50 %; BLAST window size, 1000 bp, and step size, 200 bp). In silico DDH values among the *Bacillus* whole genomes were estimated using the server-based Genome-to-Genome Distance Calculator (GGDC, ver. 2.1) (http://ggdc.dsmz.de/distcalc2.php) (Meier-Kolthoff, et al. 2013, Auch, et al. 2010), with BLAST+ for genome alignments (Camacho, et al. 2009). The overall relatedness of ANI and in silico DDH among the whole genomes were visualized as heat-map and hierarchical clustering using GENE-E (Gould, http://www. broadinstitute. org/cancer/software/GENE-E/).

III-2-5. Phylogenetic analysis based on 16S rRNA and whole genomes

To analysis evolutionary relationships among *B. subtilis* strains, the 16S rRNA gene sequences of *B. subtilis* in Table III-1 and its closely related type species were aligned using Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu) aligner (Nawrocki, et al. 2007). A phylogenetic tree was constructed using the neighbor-joining (NJ) method within the PHYLIP software (version 3.695, Felsenstein,

2002).The resulting tree topology was evaluated using a bootstrap analysis based on 1000 resampled datasets within the PHYLIP package.

To confirm a genome-based phylogenic analysis, core genes were extracted from the whole genomes of 99 *B. subtilis*-related strains using the USEARCH program with 90% sequence identity cut-off in BPGA ver.1.2 (Chaudhari, et al. 2016). The concatenated amino acid sequences of a total of 308 core genes were aligned using MUSCLE (version 3.8.31) (Edgar, et al. 2004) in BPGA and misaligned regions were removed. The neighbor-joining (NJ) tree was constructed using MEGA 7.0 (Kumar, et al. 2016) with 1000 bootstrap replications.

The presence/absence of orthologous genes in different genomes were plotted using GENE-E, with one minus Pearson correlation distances for clustering of rows (genes) and columns (genomes).

III-2-6. COG protein function analysis and KEGG pathway analysis

To investigate the relatedness among *B. subtilis* HRBS 10TDI13 and D12-5-related strains based on molecular phenotypes, Function analysis about Clusters of Orthologous Groups (COG) family by searching against the COGs database was performed using USEARCH (Edgar, 2010) program with 50% sequence identity cut-

off. A table including presence/absence of COG in the different genomes was plotted using GENE-E with one minus Pearson correlation distances for clustering of rows (gene) and columns (genomes). Ratio (%) of genes assigned to each COG functional category were represented as graph of radial type for functional comparison of the gene contents in genomes of *B. subtilis* groups. To perform KEGG Orthology (KO) functional annotation, the predicted proteins of each genome were submitted to BlastKOALA (http://www.kegg.jp/blastkoala/) (Kanehisa, et al. 2016). The results were then submitted to Mapper of Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa, et al. 2000) to reconstruct metabolic pathways. Metabolic and regulatory pathways were generated using the iPath v2 module based on KEGG annotation of genes detected from sequencing.

III-2-7. Data deposition

The whole genomes of strains 10TDI13 was deposited at GenBank. Accession numbers are listed in Table III-1.

III-3. Results and Discussion

III-3-1. Genome sequencing and general features of *B. subtilis*

B. subtilis 10TDI13 was sequenced in this study using the Illumina and Pacbio platform system. The general features of the 10TDI13 newly sequenced and 106 published genomes of *B. subtilis* summarized in Table III-1.

All the studied *B. subtilis* genomes had an average length of 4.11 Mb. *B. subtilis* GB03 genomes were the smallest (3.85 Mb) and *B. subtilis* S1-4 was the largest genome (4.45 Mb). The GC contents ranged from 42.90% to 46.60%. Total number of genes are from 3,702 to 4,004 and the number of protein coding sequences varied from 3,033 to 4,538.

Genome quality check were assessed by CheckM program was conducted using gene markers specific to taxon of *B. subtilis*. Except for BEST7613, B4122, PS216, all genomes were assessed that satisfied the criteria required to be considered a near-complete genome with low contamination ($\geq$90 % completeness value and $\leq$5 % contamination value). BEST7613, B4122, PS216 genome was showed high contamination value (100.46%, 10.51%, 5.27%), respectively. Among four genomes of *B. subtilis* 168, only one genome which registered latest was used. The genomes

which have not enough data information such as number of protein coding sequences, 16S rRNA sequence, and having more than 1000 unique gene are excluded in this study. In addition, the genomes, which harbor more than 10% pseudogenes or have insufficient information compared other genomes, was also excluded because including that genomes, which makes it difficult to investigate, could not clustered with the same species taxon in GOC or KEGG analysis.

B. subtilis consists of three subspecies (*B. subtilis* subsp. *subtilis*, *B. subtilis* subsp. *inaquosorum*, and *B. subtilis* subsp. *spizizenii*) (Nakamura, et al. 1999). To clarify the taxonomic relationship of the *B. subtilis* strains and relatively closed type species belonging to *B. subtilis* group, 16S rRNA sequence was used.

III-3-2. Phylogenetic analysis based on 16S rRNA

A phylogenetic tree using the NJ algorithm based on the 16S rRNA gene sequences of strains 10TDI13, closely related *Bacillus* strains and *Bacillus* type species showed that the strains 10TDI13 were closely clustered together with *B. subtilis*. However, the phylogenetic tree of *B. subtilis* strains comprised of three subspecies, *B. subtilis* subsp. *subtilis*, *B. subtilis* subsp. *inaquosorum*, and *B. subtilis* subsp. *spizizenii* displayed that the three *Bacillus* subspecies were not differentiated by their 16S rRNA gene sequences. In addition, the 16S rRNA sequences similarity among *Bacillus* species were also very high so that the strains belonging to *B. subtilis* and 11 *Bacillus* type species did not clearly divided into different phylogenetic lineages (Figure III-1).

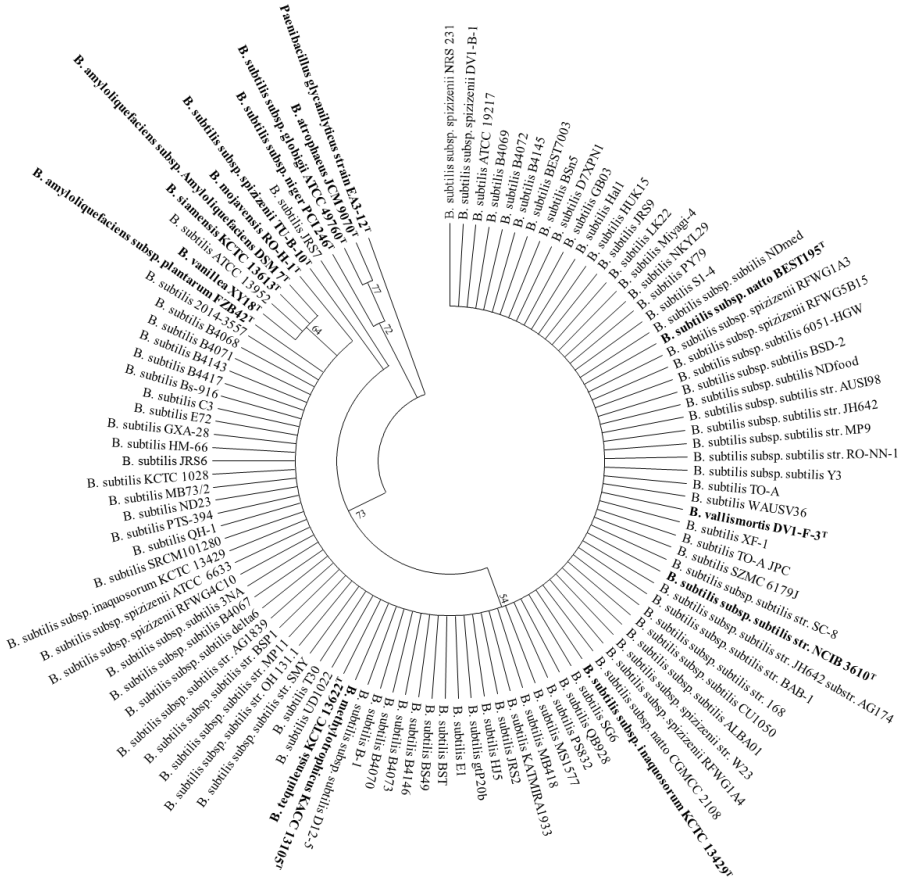


Figure III-1. A Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships of strain 10TDI13, closely related *Bacillus* type strains, and closely related *B. subtilis* strains with whole genome sequences available in GenBank. The strain names as described in GenBank were used in the tree and the type strains were highlighted in bold. Bootstrap values are shown on nodes in percentages of 1,000 replicates, when only values over 70 %. *Paenibacillus glycanilyticus* strain EA3-12 ^{T} (JF49641) was used as outgroup. Bar, 0.01 changes per nucleotide position.

III-3-3. Relatedness based on ANI and in silico DDH

Richter & Rosselló-Móra suggested that approximately 95-96% could be used as the ANI cut-off value corresponding to 70% DDH level, the gold standard of the prokaryotic species delineation (Goris, et al. 2007, Richter, et al. 2009, Tiedje, 2007), because ANI value has been recognized as a robust means to reflect the degree of evolutionary distance between the compared genomes (Goris, et al. 2007, Konstantinidis, et al. 2007).

ANI values among all *B. subtilis*-related 106 strains in Table III-1 were calculated using their whole genomes. The ANI results displayed that the *B. subtilis*-related strains can be classified into at least eight different species containing three *B. subtilis* subspecies belonging to the genus *B. subtilis* (Figure III-2). Interestingly, the hierarchical clustering based on the ANI values was totally difference with the tree topology based on 16S rRNA gene sequences shown in Figure III-1. Strain 10TDI13 shared high ANI values of 98.74% with the type strains of *B. subtilis* subsp. *subtilis* NCIB3610, but the strain shared 92.96% and 93.04% ANI values with each type strain of *B. subtilis* subsp. *inaquosorum* KCTC13429 and *B. subtilis* subsp. *spizizenii* TU-B-10, respectively.

In silico DDH analysis also demonstrated that the *B. subtilis*-related strains can be classified into at least eight different species of the genus *B. subtilis* and the topology of hierarchical clustering based on the in silico DDH values was identical with that based on the ANI values shown in Figure III-3. Each of the eight lineages clearly classified by both ANI and DDH values were suggested that they could be represent a different new species of the genus *B. subtilis*.

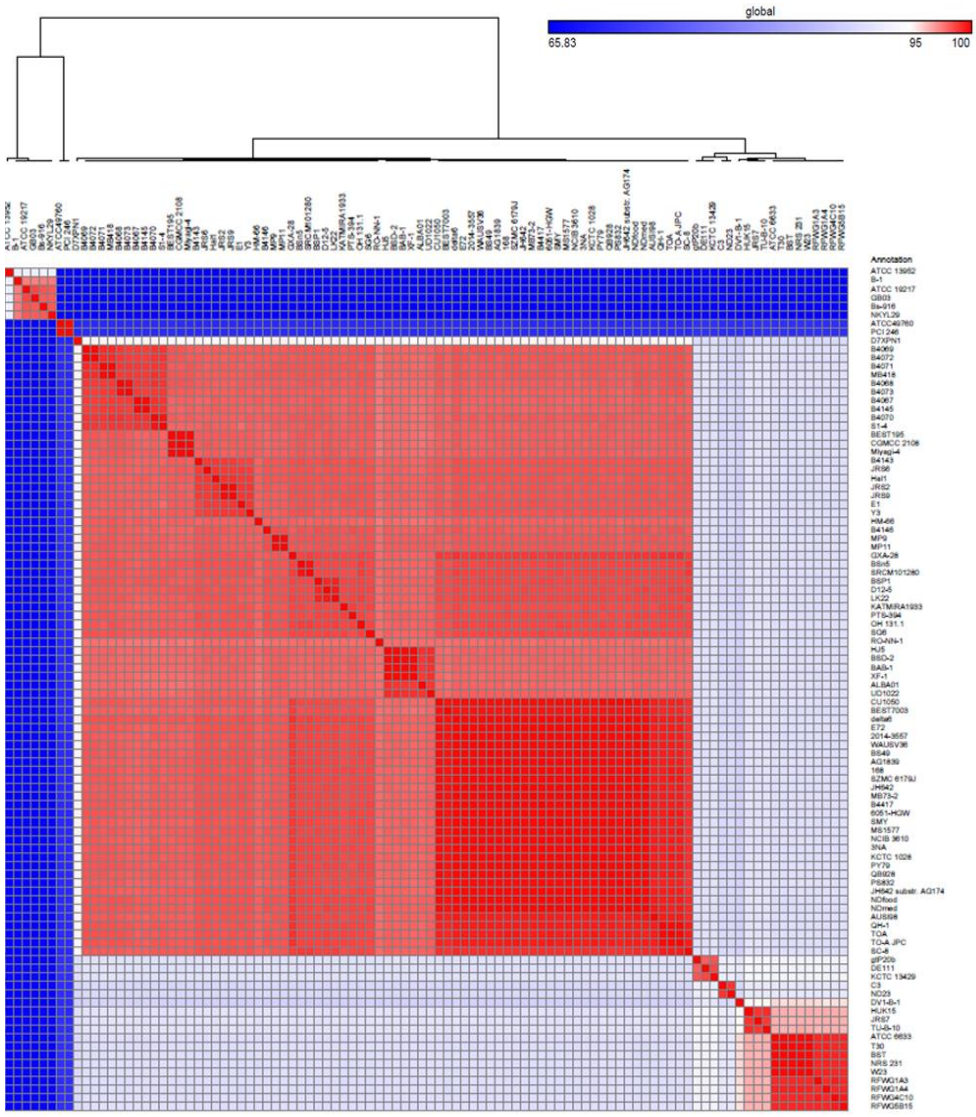

global
65.83
95
100
Annotation

Figure III-2. Heat-map showing relatedness among *B. subtilis* and its related strains based on ANI values (%). The strain names as described in GenBank were given as row and column labels and the type strains of *B. subtilis* subspecies were highlighted in bold. Squares corresponding to less than 95% ANI values in the heat-map are colored in blue, indicating probably the different species. The strains corresponding to red squares with greater than 95% ANI values may indicate the same species. Color intensity fades as the ANI values approach 95%. Hierarchical clustering on the top is represented by a dendrogram, constructed by a simple linkage of the ANI values.

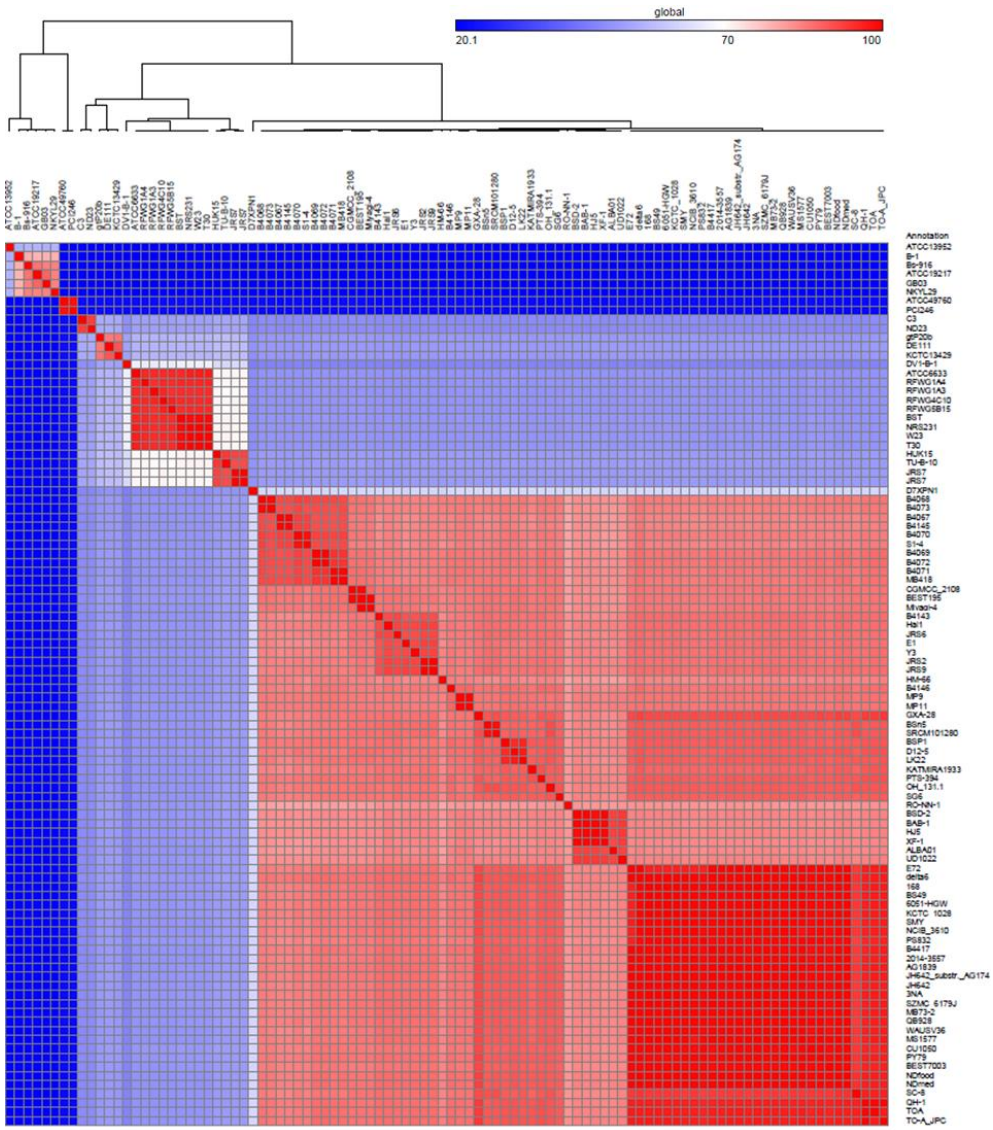

global
20.1
70
100
Annotation

Figure III-3. Heat-map showing relatedness among *B. subtilis* and its related strains based on in silico DDH values (%). The strain names as described in GenBank were given as row and column labels and the type strains of *B. subtilis* subspecies were highlighted in bold. Squares corresponding to less than 70% in silico DDH values in the heat-map are colored in blue, indicating probably the different species. The strains corresponding to red squares with greater than 70% in silico DDH values may indicate the same species. Color intensity fades as the in silico DDH values approach 70%. Hierarchical clustering on the top is represented by a dendrogram, constructed by a simple linkage of in silico DDH values.

III-3-4. Phylogenetic analysis based on whole genomes

Because the 16S rRNA gene of *Bacillus* genus is highly conserved, it is not appropriate method to differentiate each group, divided by ANI and DDH value. To gain insight into the natural history of *Bacillus* based on the complete genome sequence, we performed a phylogenetic reconstruction using the core genes of 99 *Bacillus* strains and the type strains of *Bacillus* group.

The phylogenetic tree based on 308 core genes showed a large discrepancy with the phylogenetic tree based on 16S rRNA gene sequences, however, it was fully consistent with the hierarchical clustering based on ANI and in silico DDH values, which supporting the idea that the eight lineages should be speciated into different phylogenetic lineages (Figure III-4). *B. subtilis* subsp. *inaquosorum* and *B. subtilis* subsp. *spizizenii* were clearly clustered and were separated from *B. subtilis* subsp. *subtilis*. Strains C3, ND23 and strain D7XPN1 were formed new lineages, respectively, which were distinct from other known three *B. subtilis* subspecies lineage.

The type strain of *B. subtilis* subsp. *spizizenii* TU-B-10 and two strains (HUK15 and JRS7) were also clearly distinguished from the strains of Lineage V (DV1-B-1, RFWG1A4, RFWG4C10, RFWG1A3, RFWG5B15, ATCC6633, NRS 231, BST,

T30, and W23) with boundary-value in silico DDH values, which suggested that they could be the same species. The phylogenetic tree based on core genes also suggested that each of eight lineages can represent a different species of the genus *Bacillus*.

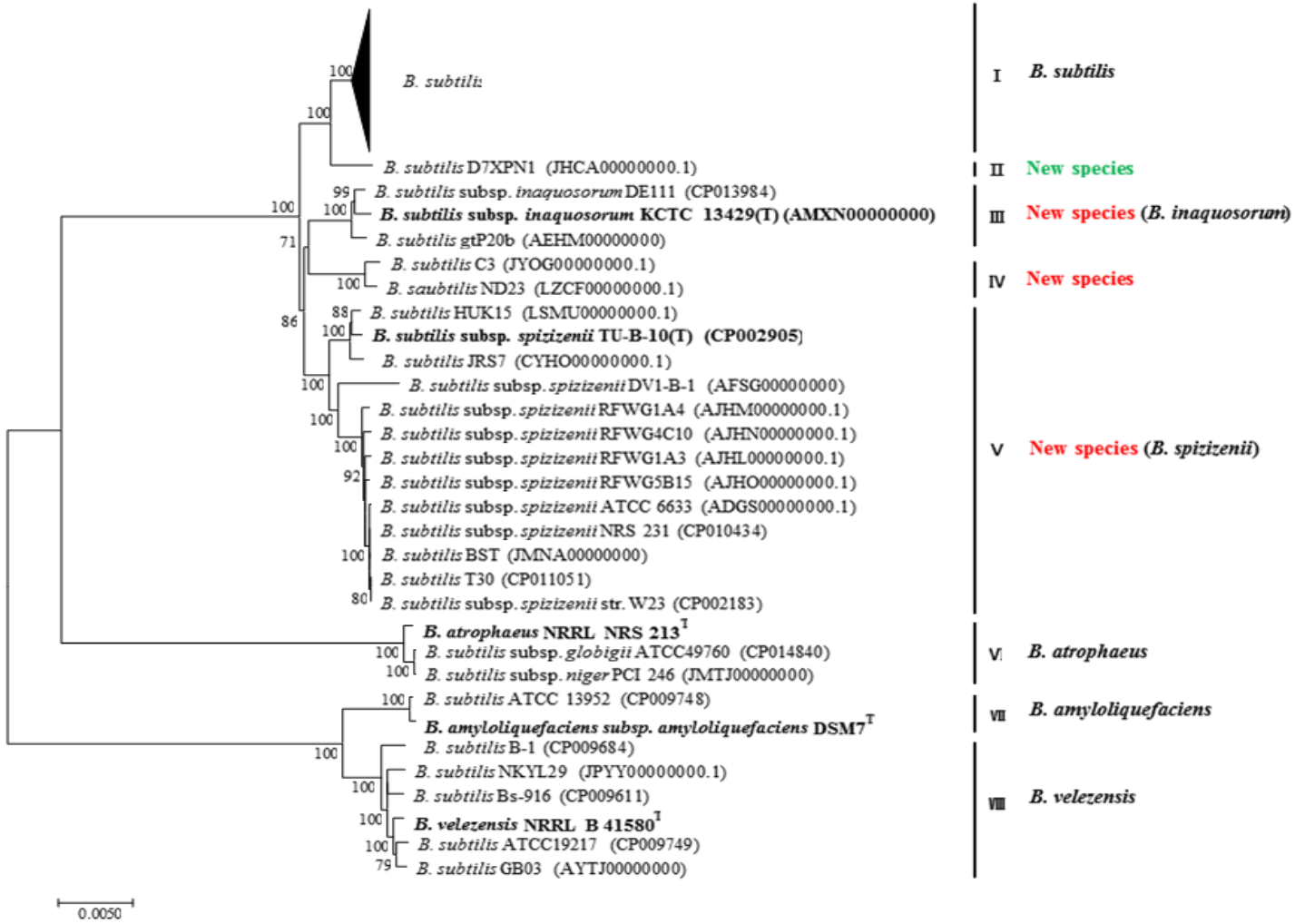

B. subtilis
B. subtilis D7XPN1 (JHCA00000000.1)
B. subtilis subsp. inaquosorum DE111 (CP013984)
B. subtilis subsp. inaquosorum KCTC 13429(T) (AMXN00000000)
B. subtilis gtP20b (AEHM00000000)
B. subtilis C3 (JYOG00000000.1)
B. saubtilis ND23 (LZCF00000000.1)
B. subtilis HUK15 (LSMU00000000.1)
B. subtilis subsp. spizizenii TU-B-10(T) (CP002905)
B. subtilis JRS7 (CYHO00000000.1)
B. subtilis subsp. spizizenii DV1-B-1 (AFSG00000000)
B. subtilis subsp. spizizenii RFWG1A4 (AJHM00000000.1)
B. subtilis subsp. spizizenii RFWG4C10 (AJHN00000000.1)
B. subtilis subsp. spizizenii RFWG1A3 (AJHL00000000.1)
B. subtilis subsp. spizizenii RFWG5B15 (AJHO00000000.1)
B. subtilis subsp. spizizenii ATCC 6633 (ADGS00000000.1)
B. subtilis subsp. spizizenii NRS 231 (CP010434)
B. subtilis BST (JMNA00000000)
B. subtilis T30 (CP011051)
B. subtilis subsp. spizizenii str. W23 (CP002183)
B. atrophaeus NRRL NRS 213T
B. subtilis subsp. globigii ATCC49760 (CP014840)
B. subtilis subsp. niger PCI 246 (JMTJ00000000)
B. subtilis ATCC 13952 (CP009748)
B. amyloliquefaciens subsp. amyloliquefaciens DSM7T
B. subtilis B-1 (CP009684)
B. subtilis NKYL29 (JPYY00000000.1)
B. subtilis Bs-916 (CP009611)
B. velezensis NRRL B 41580T
B. subtilis ATCC19217 (CP009749)
B. subtilis GB03 (AYTJ00000000)
0.0050
I B. subtilis
II New species
III New species (B. inaquosorum)
IV New species
V New species (B. spizizenii)
VI B. atrophaeus
VII B. amyloliquefaciens
VIII B. velezensis

Figure III-4. Phylogenetic tree of *B. subtilis* and its related strains reconstructed using the concatenated amino acid sequences of 308 core genes. Bootstrap values are shown on nodes in percentages of 1000 replicates, when only values over 70 %. The strain names as described in GenBank were used in the tree and the type strains were highlighted in bold. Bar, 0.005 substitutions per site.

III-3-5. The pan- and core-genomes analysis

The pan genome analysis for 99 *B. subtilis* was performed and is shown in Figure III -5 and Figure III-6. The pan genome of *B. subtilis* consists of core (308), dispensable (14,632), and specific (4,386) gene pool (Figure III-5A, Figure III-6A). The results of pan-genome revealed that core to pan ratio of *B. subtilis* is very low (1.59%) while unique to pan ratio is somewhat high (22.69%).

To study the functional and metabolic features of four lineages classified as *B. subtilis*, the pan-genome analysis of total 91 *B. subtilis* strains except for 8 strain belonging to *B. artrophaeus, B. amyloliquefaciens*, and *B. velenzensis* were conducted. Lineage II (D7XPN1) was also excluded because this lineage has only one genome, which is not enough to figure out the feature of that lineage (Figure III-5B, Figure III-6B). The pan genome analysis of the four *Bacillus* lineages based on the orthologous genes (OGs) was conducted and is shown in Figure III-7A. The pan gnome of the four lineages consist of 4,270 OGs, including core genome OGs, dispensable genome OGs which is shared by at least two genomes, and specific OGs. The number of core genome OGs was 1,339. The core genome OGs represents 90% of all proteins present in each genome. Lineage III harbors the highest number of singletons (271), followed by Lineage IV, which harbors 167 singletons. Lineage I

and lineage V have with 15 and 40 OGs, respectively. Lineage III, IV, and V share the highest protein sequences (937 genes) which corresponds to 21.9% of all present proteins in the pan genome. However, their unique genes in lineage I, III, IV, and V were 3, 31, 25, and 6, respectively (Figure III-7B). Each *Bacillus* lineage contained a number of unique proteins, however the majority of these contained genes encoded hypothetical proteins. Although, most unique genes of each lineage were identified as hypothetical protein, the genes contained in each lineage were found only in the respective lineage (Table III-2). A previous study found that a subtilin synthesis system was exclusively presence in subspecies *spizizenii* and it could even distinguish subspecies *spizizenii* from subspecies *inaquosorum* (Yi, et al. 2014). In contrast, a subtilin synthesis symtem did not contain in the list of unique genes. It is because numbers and types of unique genes affiliated to each lineage may vary depending on the number of genome used and tools used for pangenome analyze. The *B. subtilis* consisting of three subspecies, *B. subtilis* subsp. *subtilis*, *B. subtilis* subsp. *inaquosorum*, and *B. subtilis* subsp. *spizizenii* are not clearly differentiated on the basis of their phenotypic properties and 16S rRNA gene sequences because their phenotypic features and 16S rRNA sequence are extremely similar. Therefore, their

unique genes can be used as maker gene to distinguish and identify the strains belonging to different lineages.

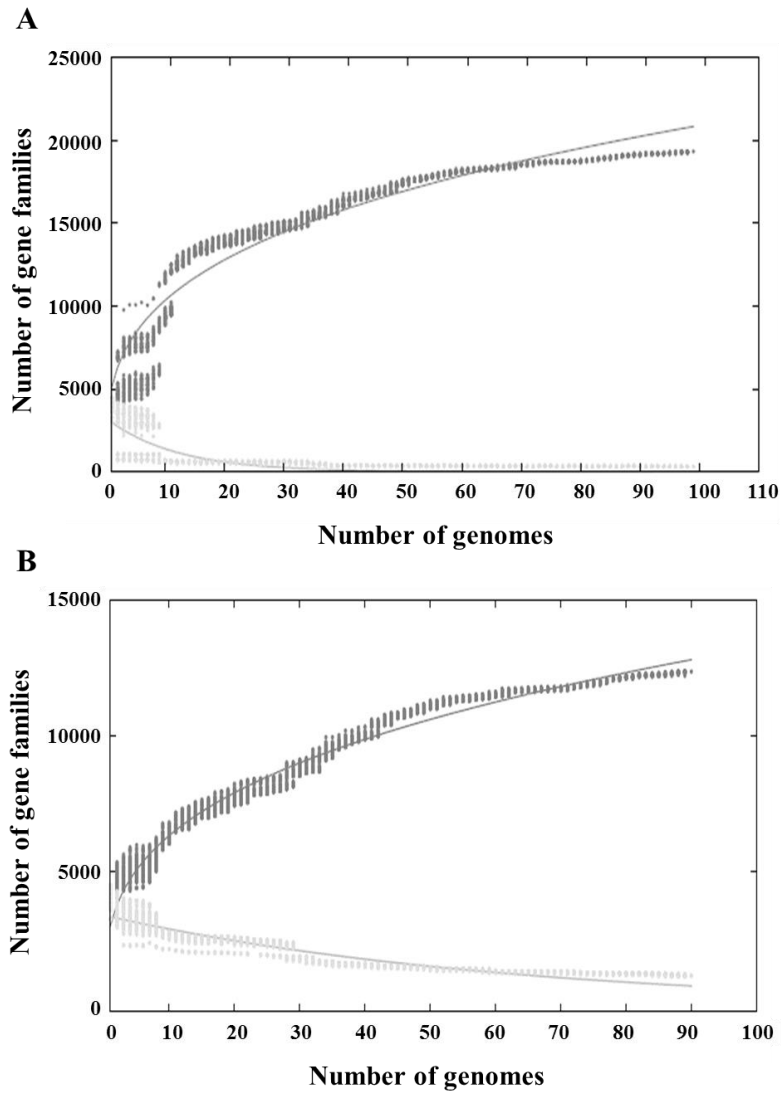


Figure III-5. The *B. subtilis* pan- and core- genome plot assigned to 99 genomes affiliated to *B. subtilis* in GenBank (A) and 90 genome belonging to *B. subtilis* lineages (B). Black lines represent the number of cumulative genes and grey lines represent the number of conserved genes as the number of genomes added.

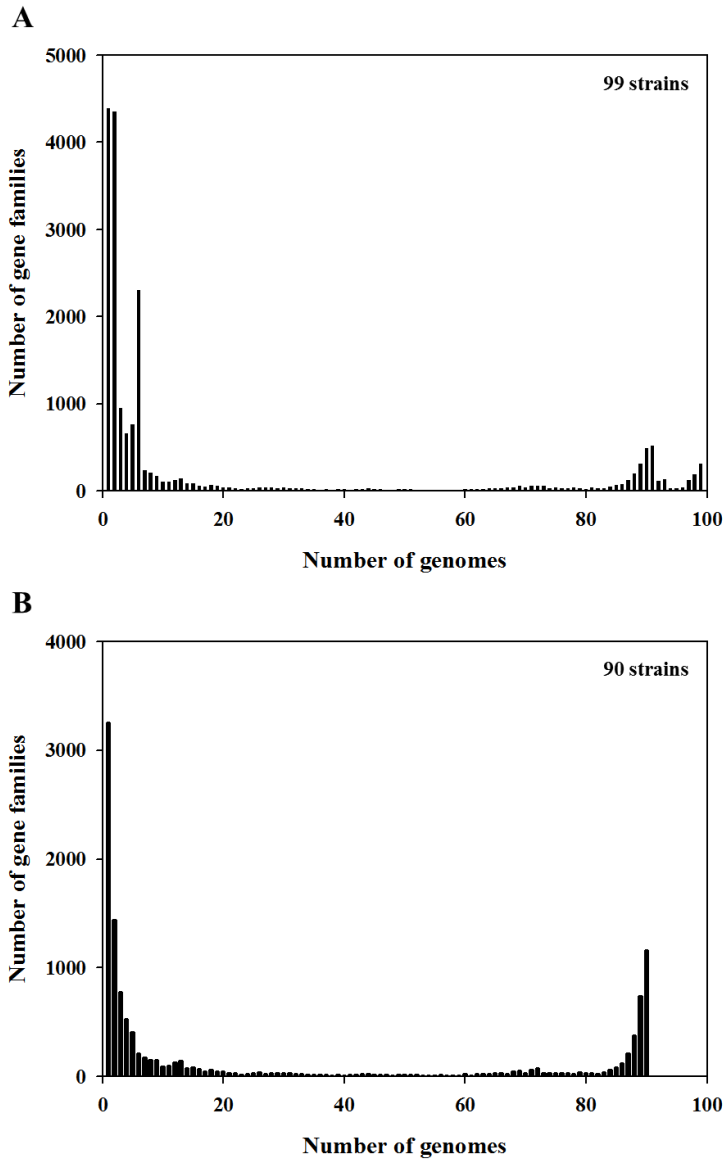


Figure III-6. Histogram of the number of genomes sharing number of gene families assigned to 99 genomes affiliated to *B. subtilis* in GenBank (A) and 90 genome belonging to *B. subtilis* lineages (B).

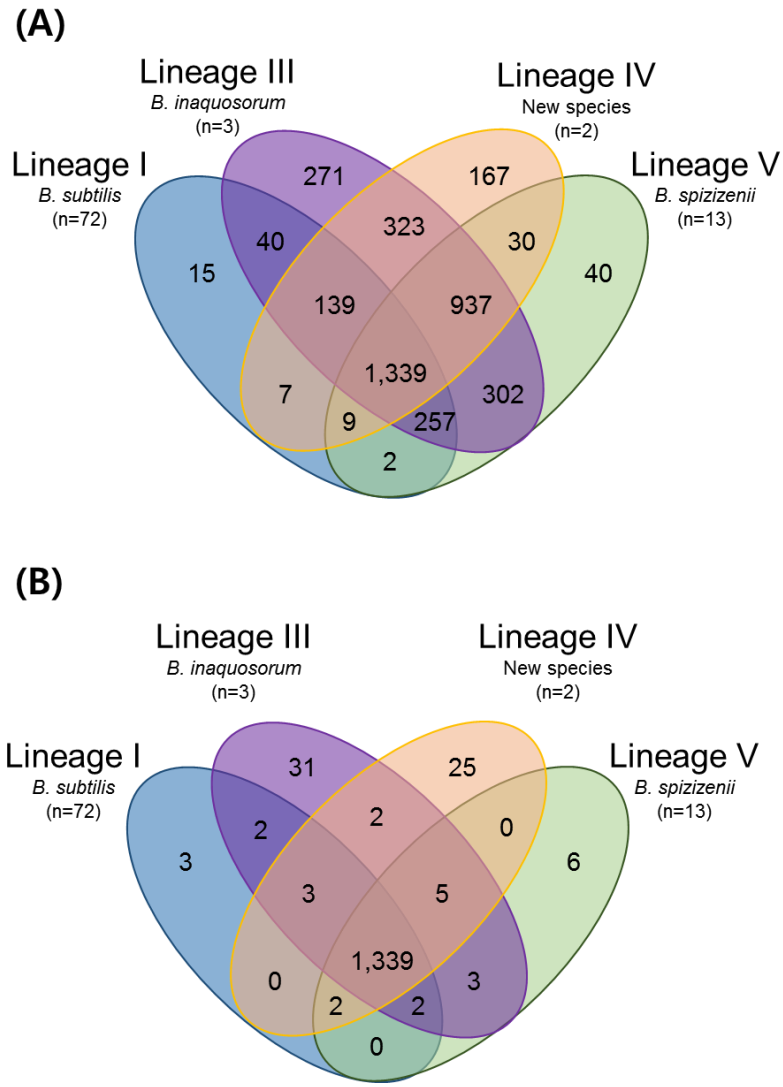


Figure III-7. Venn diagram showing the number of core gene (in the center) and unique genes in each *Bacillus* lineages. Core- and unique- genes predicted from four *Bacillus* lineages based on the orthologous genes (OGs) (A) and lineage unique genes (B).

Table III-2. List of unique genes identified in four *B. subtilis* lineages

Feature name	Lineage				Product
	I	III	IV	V	
	n=72	n=3	n=2	n=13	
WP_064671110.1	+				hypothetical protein
WP_004399338.1	+				hypothetical protein
WP_017697165.1	+				hypothetical protein
WP_003240121.1		+			3-hydroxybutyryl-CoA dehydrogenase
WP_003240117.1		+			acyl carrier protein
WP_019257687.1		+			acyl-CoA dehydrogenase
WP_019257580.1		+			dehydrogenase
WP_019257686.1		+			hypothetical protein
WP_003240124.1		+			hypothetical protein
WP_019257552.1		+			hypothetical protein
WP_019260034.1		+			hypothetical protein
WP_003240507.1		+			hypothetical protein
WP_050816612.1		+			hypothetical protein
WP_003238417.1		+			hypothetical protein
WP_060399761.1		+			hypothetical protein
WP_019258443.1		+			hypothetical protein
WP_019259389.1		+			hypothetical protein
WP_060399021.1		+			hypothetical protein
WP_019258800.1		+			hypothetical protein
WP_019258667.1		+			hypothetical protein
WP_003240100.1		+			hypothetical protein
WP_060398160.1		+			hypothetical protein
WP_019257532.1		+			hypothetical protein
WP_060398003.1		+			MerR family transcriptional regulator

WP_019257680.1	+		MFS transporter
WP_019257681.1	+		non-ribosomal peptide synthetase
WP_060398083.1	+		non-ribosomal peptide synthetase
WP_003240114.1	+		polyketide synthase
WP_019257691.1	+		polyketide synthase
WP_060398086.1	+		polyketide synthase
WP_019257690.1	+		polyketide synthase
WP_019257682.1	+		serine hydrolase
WP_060398734.1	+		TetR family transcriptional regulator
WP_019257684.1	+		thioesterase
KJJ40288.1		+	2-dehydropantoate 2-reductase
KJJ41560.1		+	ABC transporter
KJJ41557.1		+	ABC transporter
KJJ40289.1		+	acetoacetate decarboxylase
OBA04470.1		+	alcohol dehydrogenase AdhP
OBA01886.1		+	cyclic nucleotide-binding protein
KJJ41559.1		+	dehydrogenase
OBA07034.1		+	esterase
OBA04984.1		+	hypothetical protein
KJJ41554.1		+	hypothetical protein
OBA01888.1		+	hypothetical protein
KJJ41555.1		+	hypothetical protein
KJJ40560.1		+	hypothetical protein
KJJ43037.1		+	hypothetical protein
KJJ41700.1		+	hypothetical protein
KJJ41701.1		+	hypothetical protein
KJJ42375.1		+	hypothetical protein
OBA09095.1		+	hypothetical protein
KJJ42336.1		+	hypothetical protein
KJJ43322.1		+	hypothetical protein

KJJ41903.1	+		hypothetical protein
KJJ40846.1	+		hypothetical protein
OBA01887.1	+		lantibiotic dehydratase
KJJ40290.1	+		LysR family transcriptional regulator
OBA01881.1	+		transcriptional regulator
WP_014113058.1		+	alanine acetyltransferase
WP_003220146.1		+	AraC family transcriptional regulator
WP_003221744.1		+	AraC family transcriptional regulator
WP_019714863.1		+	cell division protein FtsW
WP_061187596.1		+	DNA-binding protein
WP_044429390.1		+	transporter

[a]Feature, or putative feature, was assigned based on BLASTp homology of encoded ORFs to known proteins.

III-3-6. Ratio (%) of genes assigned in each COG category

Genome comparison between closely related bacteria may provide valuable insights into the molecular mechanisms conferring them their physiological and phenotypic properties. However, genome comparisons between very distant bacteria can draw erroneous conclusions caused by many other different genes rather than the presence/absence or difference in gene numbers of one or two genes. To understand the functional roles of the genes that constitute the core and pan-genome, I analyzed the COG (Clusters of Orthologous Groups) distribution by assigned in 4 lineages (lineage I, III, IV, and V). The proportion of proteins in each COG category was plotted versus COG function (Figure III-8). I observed significant differences in the COGs categories of 4 lineages. Several expected features arose from this analysis, such as the predominance of gene in COG category R (general functional prediction only) in all lineages and especially, it showed the highest value in lineage V. Previous study found that several core genes of the COG R and S are conserved across the various *Bacillus*, isolated from a wide range of environment with some clearly being involved in the sporulation process, including spmA, spmB, yaaT, spoIVFB, spoVB, spoVR, and others (Alcaraz, et al. 2010). COG categories analysis showed that the

distribution between lineage I and lineage IV were similar in COG category C, J, K, S, T, and U.

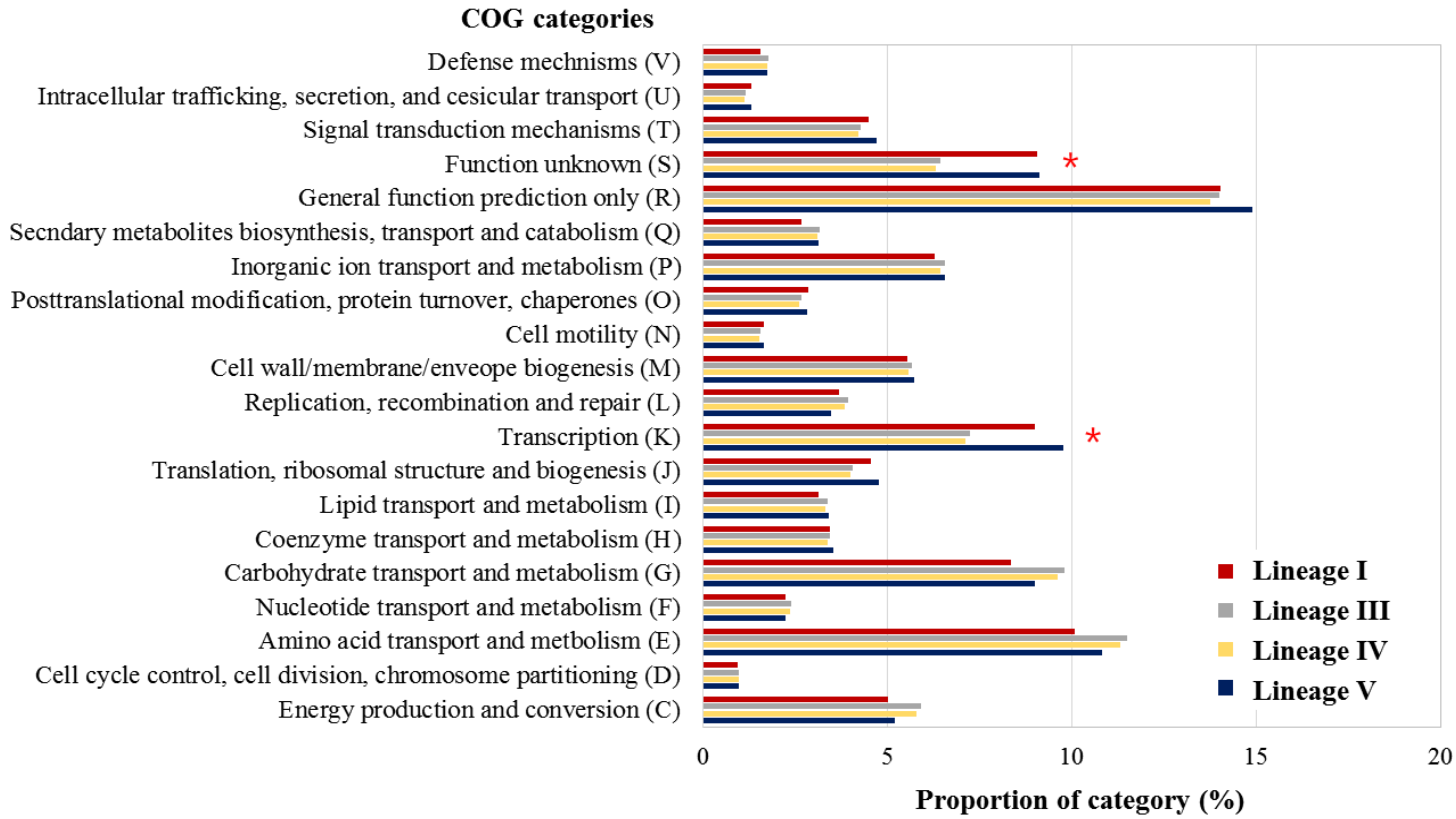


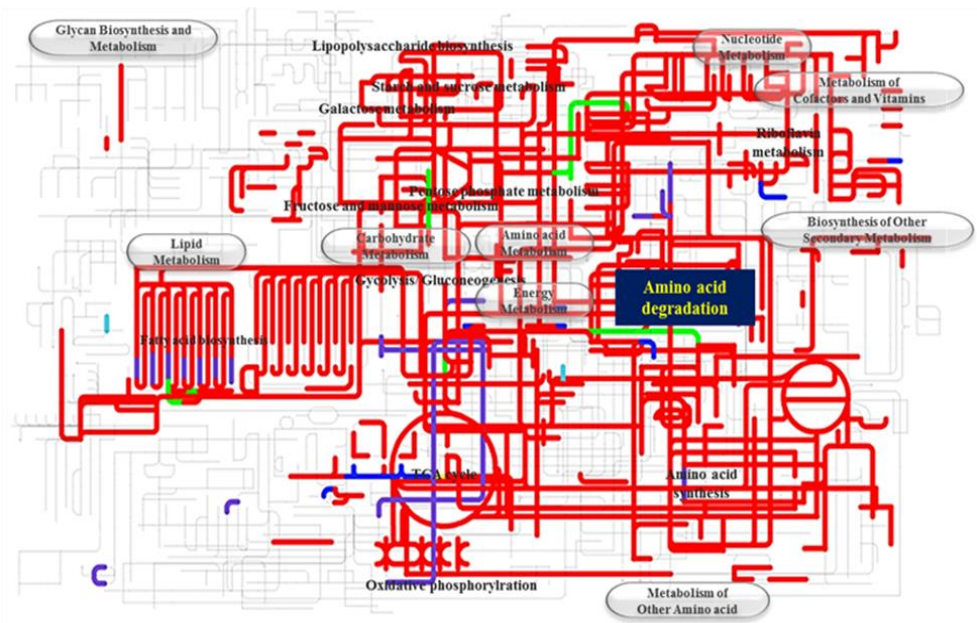
Figure III-8. Comparison of clusters of orthologous group (COG) distribution as a percentage of total genes assigned to COGs of four *Bacillus* lineages.

III-3-7. Metabolic and regulatory pathways in *B. subtilis*

The metabolic and regulatory pathways were generated using the iPath v2 module based on KEGG annotation of core genes detected from each lineage (I, III, IV, and V) of *B. subtilis* (Figure III-9). The metababolic pathway of core genes showed that all *B. subtilis* lineages have very similar pathway. All *B. subtilis* strains have genes associated with carbohydrate metabolism including pentose phosphate metabolism, fructose and mannose metabolism, starch and sugar metabolisms, glycolysis, gluconeogenesis, and TCA cycle. In addition, the strains can also be able to metabolize lipid, amino acid, and vitamin metabolism. These pathways may be essential and useful to metabolize and synthesize molecules. It is assumed that the *Bacillus* species can survive and become dominant during doenjang fermentation, because the metabolic pathways are conserved in *Bacillus* species. The regulatory pathways related with cell motility (chemotaxis and flagella assembly) and translation (ribosome and aminoacyl-tRNA biosynthesis) were present in all strains. In particular, lineage I (*B. subtilis*) has unique genes associated with amonoacyl-tRNA biosynthesis and bacterial secretion system and lineage III (*B. inaquosorum*)

has phosphotransferase system. Although, each lineage has unique regulatory pathway, their metabolic feature can not be distinguished by compare the unique pathway in each lineage.

(A)


Glycan Biosynthesis and Metabolism
Lipopolysaccharide biosynthesis
Nucleotide Metabolism
Metabolism of Cofactors and Vitamins
Galactose metabolism
Riboflavin metabolism
Pentose phosphate metabolism
Fructose and mannose metabolism
Biosynthesis of Other Secondary Metabolism
Lipid Metabolism
Carbohydrate Metabolism
Amino acid Metabolism
Glycolysis/Gluconeogenesis
Energy Metabolism
Amino acid degradation
Fatty acid biosynthesis
TCA cycle
Amino acid synthesis
Oxidative phosphorylration
Metabolism of Other Amino acid

(B)

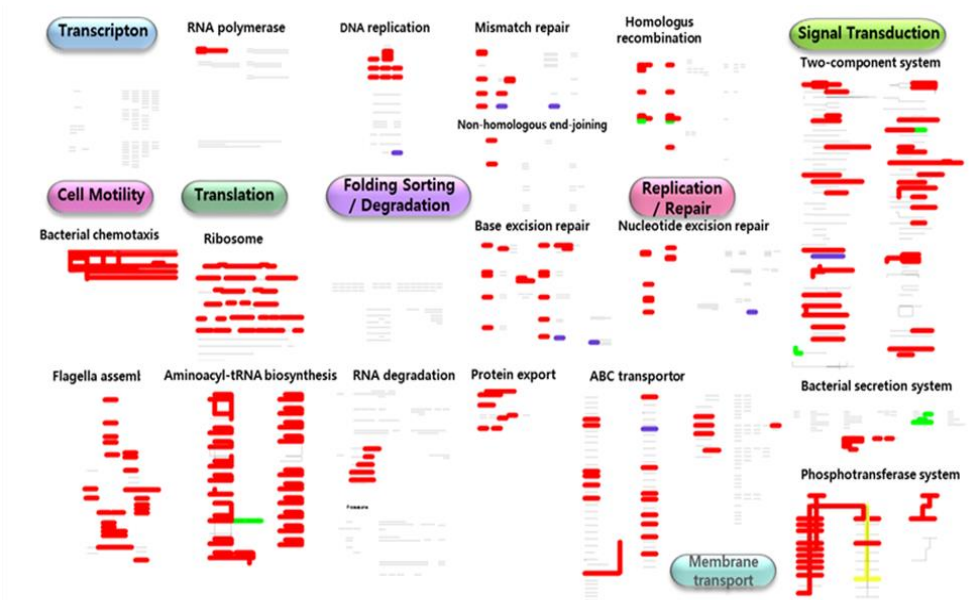

Transcripton
RNA polymerase
DNA replication
Mismatch repair
Homologus recombination
Signal Transduction
Two-component system
Non-homologous end-joining
Cell Motility
Translation
Folding Sorting / Degradation
Replication / Repair
Bacterial chemotaxis
Ribosome
Base excision repair
Nucleotide excision repair
Flagella assem
Aminoacyl-tRNA biosynthesis
RNA degradation
Protein export
ABC transportor
Bacterial secretion system
Phosphotransferase system
Membrane transport

Figure III-9. Metabolic (a) and regulatory (b) pathways in *B. subtilis*. Pathways were generated using the iPath v2 module based on KEGG annotation of genes detected from sequencing. Red line (core) represent the pathways exist in B. subtilis strains belonging to all lineages. Purple lines represent accessory genes that are exist in some but not all lineages. Green, yellow, sky blue, and blue line represent genes that are exist in lineage I, III IV, and V, respectably.

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Chapter IV.

Effects of *Aspergillus oryzae* SNU-HR on microbial communities and metabolites during meju fermentation

IV-1. Introduction

Meju, fermented soybean lump, is an absolutely essential ingredient for the manufacture of traditional Korean fermented soybean food such as doenjang (soybean paste), ganjang (soy sauce), and gochujang (hot pepper paste) (Jung, et al. 2014). The skill of preparing traditional meju has been passed down the generations by empirically and domestically without a system for sterilization or aseptic fermentation (Park, et al. 2014). The process of the traditional meju is that soaked soybean is boiled, mashed, shaped the soybean into blocks, and allowing it to ferment for 6-8 weeks under the natural environment conditions (Kim, et al. 2011). The manufacture of meju is generally started in November, when the air is dry and cold, so that the growth of bacteria inhabiting a natural environment is suspend (Kim, et al. 1997). Therefore, only a few microorganisms from natural environment may be inoculated into meju, however the bacterial and fungal communities in meju could increase and be diversified due to the microorganisms derived from dried rice straw and the facility (Yoo, et al. 1998).

The bacteria and fungi are responsible for the hydrolysis of major ingredient protein, lipid, carbohydrate, and flavonoid glycoside in meju during fermentation, resulting

in the characterizations and flavors of meju. Accordingly, meju quality affects definitely into the quality of that traditional fermented food (Kwon, et al. 2011, Jung, et al. 2014).

The physicochemical and functional properties of meju can be various depending on soybean, geographical location, microorganisms, and fermenting time, because meju is generally manufactured in a traditional way in homes using different types of processes (Lee, et al. 2010). Likewise, the conditions of traditional meju fermentation are so various that it is difficult to standardize and impracticable to commercialize.

One way to resolve these problems, koji, made of soy bean and wheat inoculated with fungi such as *Aspergillus oryzae*, has been widely used to initiate fermentation in the food industry. The koji could curtail meju fermentation period with protease, amylase, and lipase produced by fungi. Moreover, the process using koji is feasible to commercialize with uniform qualities. Similar with traditional meju, microorganisms inoculated as starter into meju are responsible for meju quality and the meju prepared by different starters exhibit significant differences in sugar, amino acid, isoflavonoid metabolites depend on the starter.

A majority of meju studies were conducted to characterize and evaluate the microbial community structure or observe their microbiota changes during the meju fermentation period of meju prepared with different starters (Jung, et al. 2014; Yoo, et al. 1998) by both culture-dependent or culture-independent method such as denaturing gradient gel electrophoresis (DGGE) analysis (Lee, et al. 2010) and pyrosequencing (Kim, et al. 2011). Furthermore, many studies have focused on nutritional qualities and metabolites of meju using various metabolomics techniques including gas spectrophotography (GC)/MS and mass spectrometry (MS) (Kang, et al. 2011, Lee, et al. 2012b).

Most studies have conducted to demonstrate partial aspect of meju features depending on various starter. However, it is very important to understand functional properties of microbial communities in meju and need to reveal the key microorganisms, which affect to tastes, flavors, and metabolites of meju, to control the quality and commercialize.

In this study, I attempted to investigate not only the changes of bacterial and fungal microbiota but also metabolites of meju during the fermentation period using a combination of culture-independent method, 454 pyrosequencing, and ^{1}H NMR technique in order to confirm the effect of adding *A. oryzae* SNU-HR isolated from

deonjang koji on meju. The results may provide better information for the overall features of the meju added *A. oryzae* SNU-HR and functional properties of major microbial communities during meju fermentation.

IV-2. Materials and Methods

IV-2-1. Preparation of starter culture

A. oryzae SNU-HR was isolated from industrial doenjang koji. *A. oryzae* SNU-HR is considered that this strain may be suitable for meju or doenjang starter candidate, because *A. oryzae* SNU-HR does not produce aflatoxin as well as cyclopiazonic acid (CPA). *A. oryzae* SNU-HR was inoculated onto PDA (potato dextrose agar) and incubated for 24 hours at 30℃. The culture was centrifuged at 5000 x g for 20 min and then lyophilized and stored at -80℃ until used.

IV-2-2. Preparation of meju samples and sampling

Two types of meju were prepared with *A. oryzae* SNU-HR at Saenggimall manufacturer (Pocheon, Korea). Approximately 150 kg soybeans were soaked in water for 18 hours at 20℃, then allowed to drain for 1 hr and boiled for 2 hours. The soybeans were then cooled to about 40℃ to inoculate starter. Whereas half the steamed soybeans were inoculated with 106 spores/g *A. oryzae* SNU-HR (TMA), the other half were not inoculated to use it for control (TMC) then were formed in blocks of which sizes were 20x20x5 cm. About 90 meju bricks were manufactured and their

outer surface was slightly dried at 20℃ for 1 day in a drying room. After 1 day, the meju bricks were hung using twist pieces of straw on the wood frame, specially made for aging meju samples, then fermented it until 35 days under natural environmental conditions. The temperature of meju fermentation was changed to 30℃ at 35 day and maintained it until the end of the fermentation.

Meju samples were collected on day 0, 1, 3, 7, 14, 21, 28, 35, 38, 42, 49, 56, and 62. As whole soybeans were boiled in the same pot and then molded, these replicates should not been considered as true biological replicates. Because the three meju samples of each group were crushed and homogenized with mixer for further experiments. All sample were stored at -80℃ for extraction of genomic DNA and metabolite analysis.

IV-2-3. pH, water content, and bacterial counting

pH values of the meju samples were immediately measured at a sample/water ratio of 1:10 (wt/wt) with pH meter. Water contents in meju were measured by weighing 5 g of homogenized samples before and after drying at 105℃ for 24 h in a dry oven. The number of viable cells of bacteria in meju was determined using a standard viable cell counting method. Briefly, 1 g of homogenized meju samples was

resuspended and serially diluted in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.2). The diluted samples were spread on tryptic soy agar (TSA, BD, USA) for the enumeration of bacteria. The agar plates were incubated at 30℃ for 1 days and the number of bacteria in meju was calculated as colony forming units (CFU) per gram.

IV-2-4. Quantitative PCR to estimate the number of bacteria and fungi

In order to estimate the 16S rRNA gene copy numbers of the total bacteria and fungi during meju fermentation using quantitative PCR (qPCR), the total genomic DNA of the pellets derived from 0.3 g of meju was extracted using a FastDNA Spin kit (MPbio, Solon, OH). Two qPCR primer sets, bac340F (5'-CCTACGGGAGGC AGCAG-3') / bac758R (5'-CTACCAGGGTATCTAATCC-3') and FR1 (5'-AICCAT TCAATCGGTAIT-3')-FF390 (5'-CGATAACGAACGAGACCT-3'), were used to measure the bacterial and fungal 16S rRNA gene copies, respectively (Juck, et al. 2000, Vainio, et al. 2000). The qPCR was performed in triplicate as described previously (Jung, et al. 2011). Two standard curves for the calculations of the bacterial and fungal 16S rRNA gene copies were generated on the basis of the number of pCR2.1 vectors (Invitrogen, USA) carrying bacterial (*Staphylococcus)*

and fungal (*A. oryzae*)16S rRNA genes derived from a saeu-jeot sample. The bacterial and archaeal 16S rRNA gene copies of each sample were calculated as described previously (Ritalahti, et al. 2006).

IV-2-5. Evaluation of protease, amylase activities

The proteolytic and amylase activity of the meju was evaluated by the quantitative disc diffusion method. One gram of each meju sample was resuspended in 9 ml PBS, and cell-free supernatants were obtained from the cultures by centrifugation (13200 rpm, 15 min). Cell-free supernatant (20 μl) was loaded on paper disk on TSA agar (Difco, USA) containing 1.5% bovine skim milk powder (Difco, USA) and the protease activities were evaluated based on clear zones forming around the paper disks after a 24h incubation at 30℃. For investigation of amylase activity, starch agar plates containing 1% soluble starch, 0.2% yeast extract, 0.1% K2HPO4, 0.15% 160 MgSO4·7H2O, 7.5% NaCl, and 1.5% agar were prepared, and 20 μl of meju supernatant was dropped onto the starch agar plates and the plates were grown at 37°C for 24 h. Amylase activities were assayed using Gram's iodine solution after a 24-h incubation at 37℃.

IV-2-6. Genomic DNA extraction and pyrosequencing

For the phylogenetic analysis of Bacteria and fungi in meju prepared using starters, total genomic DNA of meju samples was extracted using a FastDNA Spin kit (MPbio, USA). The hypervariable regions of bacterial 16S rRNA (V1–V3 variable regions) and fungi 28S rRNA (D1–D2 regions) were amplified using two primer sets BacF (5'-adaptor B-AC-9 F-3') / BacR (5'-adaptor A-X-AC-541R-3') (Lee, et al. 2012a) and FunF (5'-adaptor B-AG-LR0R -3') / FunR (5'-adaptor A-X-AG-LR3-3') (Liu, et al. 2012), where X denotes unique 7–11 barcoded sequences inserted between the 454 Life Sciences adaptor A sequence and a common linker AG (Table IV-1) (Roesch, et al. 2007). All the PCR amplifications were carried out in a C1000 thermal cycler (Bio-Rad) with a 50-μl volume containing Fh-Taq polymerase mixture (Solgent, Korea), 1 μl genomic DNA, and 20 pmol of each primer and the cycling regime was as follows: 94℃ for 5 min (1 cycle); 94℃ for 45 s, 55℃ (for bacteria) or 50℃ (for fungi) for 45 s, and 72℃ for 45 min (30 cycles); and 72℃ for 10 min (1 cycle). The PCR products were purified using a PCR purification kit (Bioneer, USA) and their concentrations were carefully measured with an ELISA reader equipped with a Take3 multivolume plate (SynergyMx, BioTek, USA). Composite

samples for pyrosequencing were prepared by pooling equal amounts (50 ng/μl) of purified PCR amplicons from each sample and then sequenced using a 454 pyrosequencing Genome Sequencer FLX Titanium system (Roche, Germany) , according to the manufacturer's instructions, by a commercial sequencing facility (Macrogen, Seoul, Korea).

Table IV-1. List of adapters, 16S rRNA primers, and barcode sequences in the PCR primer sets used in this study

Name	Sequence (5'->3')			Reference
Adapter sequence				
A dapter seqeunce	CCATCTCATCCCTGCGTGTCTCCGACTCAG			Roesch et al., 2007
B dapter seqeunce	CCTATCCCCTGTGTGCCTTGGCAGTCTCAG			Roesch et al., 2007
16S rRNA primers				
9F	GAGTTTGATCMTGGCTCAG			DeLong, 1992
541R	WTTACCGCGGCTGCTGG			DeLong, 1992
LR0R	ACCCGCTGAACTTAAGC			Liu et al., 2012
LR3	CCGTGTTTCAAGACGGG			Liu et al., 2012
Bacteria (TMC)		Fungi (TMC)		
0	ACTCGTATC	0	TGTCAGC	This study
1	CATGCTC	1	AGCGATG	This study
3	AGAGCTG	3	CTGTCTACG	This study
7	ACGTCTCTACG	7	CACACGATAG	This study
14	TATGCAC	14	TAGATAGTGCG	This study
21	TCTGCAG	21	ATGCTGAG	This study
28	AGCGATG	28	ATGTGTCTAG	This study
35	ATGCTGAG	35	CATGCTC	This study
38	TACAGCAG	38	TGACTCGAC	This study
42	ATGCAGAC	42	TGTCAGC	This study
49	AGATCGCT	49	AGCGATG	This study
56	CATGTAGC	56	CTGTCTACG	This study
62	ATCGTGTG	62	CACACGATAG	This study
Bacteria (TMA)		Fungi (TMA)		This study
0	TAGATAGTGCG	0	CATCATC	This study
1	TAGCTACG	1	ATCGCATC	This study
3	TGACTCTG	3	AGTCACTAG	This study
7	ACACTGTG	7	ATCACGTGCG	This study
14	TCGCTATC	14	ACGTCTCTACG	This study
21	ACTACACGC	21	TACAGCAG	This study
28	TGACTCGAC	28	ATCGTCTGTG	This study
35	CGTGTACTG	35	AGAGCTG	This study
38	CTGTCTACG	38	CGTGTACTG	This study
42	CAGTCTCGA	42	CATCATC	This study
49	CAGACAGAT	49	ATCGCATC	This study
56	AGCTCACTG	56	AGTCACTAG	This study
62	CACACGATAG	62	ATCACGTGCG	This study

IV-2-7. Sequencing processing and data analysis

The pyrosequencing data were processed and analyzed using RDP pyrosequencing pipeline (http://pyro.cme.msu.edu) (Cole, et al. 2014). The pyrosequencing sequence were sorted to the specific samples based on their unique barcodes, after which the barcode and primer sequences were removed. Reads with more than two undetermined nucleotides and/or read lengths shorter than 300 bp and more than 700 bp were excluded from subsequent analysis, and potential chimeric reads were discarded by using the UCHIME chimera slayer (Edgar, et al. 2011) in RDPipeline. To compare the microbial diversity among the meju samples, the bacterial and fungal reads numbers of each sample were normalized to those of the sample with the smallest number of reads (249bp) by random removal of sequencing reads using the sub.sample command of the mothur program (Schloss, et al. 2009). Operational taxonomic units (OTUs), Shannon-Weaver (Shannon, et al. 2015), and Chao1 biodiversity (Chao, 1987) indices and evenness for the normalized sequencing reads were calculated using RDPipeline at a 97% identity cutoff value.

IV-2-8. Statistical analysis

To investigate the correlations microbial communities of two meju samples, a redundancy analysis (RDA) was performed using the package 'VEGAN' (Okasanen, et al. 2016) in R programming environment (http://cran.r-project.org) on the basis of the relative abundance of bacterial and fungal taxonomic groups classified at the genus level. To investigate the correlation between pH, water contents, amylase, and protease and the respective bacterial and fungal taxonomic groups (relative abundance (%) of bacteria and fungi) during meju fermentation, Pearson correlation coefficients and P value were calculated using the SPSS statistics package ver 12.0 (SPSS, USA).

IV-2-9. Metabolite analysis

The meju metabolites including amino acids, free sugar, and organic acids during the fermentation day were analyzed in triplicate using ^{1}H NMR spectroscopy according to a modification of the methods descried previously (Lee, et al. 2009). Briefly, 0.2 g of lyophilized meju samples were dissolved in 1.4 ml 99.9% deuterium oxide (D2O, Sigma-Aldrich, USA) with 5 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, 97%, Sigma-Aldrich). After extraction in ice for 1 hr with occasional shaking, samples were centrifuged at 13,000 rpm and 4℃ for 10 min. The resulting

supernatants were transferred into 5-mm NMR tubes and ^{1}H NMR spectra were acquired using a Varian Inova 600-MHz NMR spectrometer (Varian, USA). Identification and quantification of individual metabolites from the ^{1}H NMR spectra were conducted using the Profiler module of the Chenomx NMR suite program (ver. 6.1, Chenomx, Canada) and the regions corresponding to water were removed. All metabolite concentrations in meju were calculated as μmol per gram-dry weight meju.

IV-3. Results

IV-3-1. Changes in pH and water content

Meju, typical Korean soybean lump making for doenjang, was prepared using steamed soybean. *A. oryzae* SNU-HR was cultivated and used for the starter meju preparations to address the effects of the meju starter on fermentation rate, microbial community, and metabolite production of meju. The changing pH and water content patterns during meju fermentation period of two meju samples are shown in Figure IV-1 A. The initial pH values of the meju samples were approximately 6.6, which decreased rapidly to approximately 5.1 during the early meju fermentation (0 - 7 days). The pH rapidly became more basic again, reaching 6.3 - 6.6 at 21 day, after then the pH gradually increased during the late fermentation period. The pH values finally reached approximately 6.9 in TMC, although the pH values of the starter added meju (TMA) were slightly lower than the non-starter meju (6.3).

The initial water content of meju was approximately 61% (Fig. IV-1A). During the fermentation period, water content of TMC and TMA samples steadily decreased and then eventually, after at 62 days, water content became approximately similar at around 8.5 %. The water content of TMA was 1-2 lower than TMC meju in general

during the whole fermentation period. In previous studies of the water content in the exterior and interior parts of meju sample, a decline in water contents of the exterior regions was very fast, while interior regions showed a steady decrease during the drying period at 40℃ for 20 h (Jung, et al. 2014). When the water contents were measured without distinction of the exterior and interior regions, the profiles of the water content in meju were similar with those demonstrated by several authors (Hur, et al. 1991, Yoo, et al. 1998)

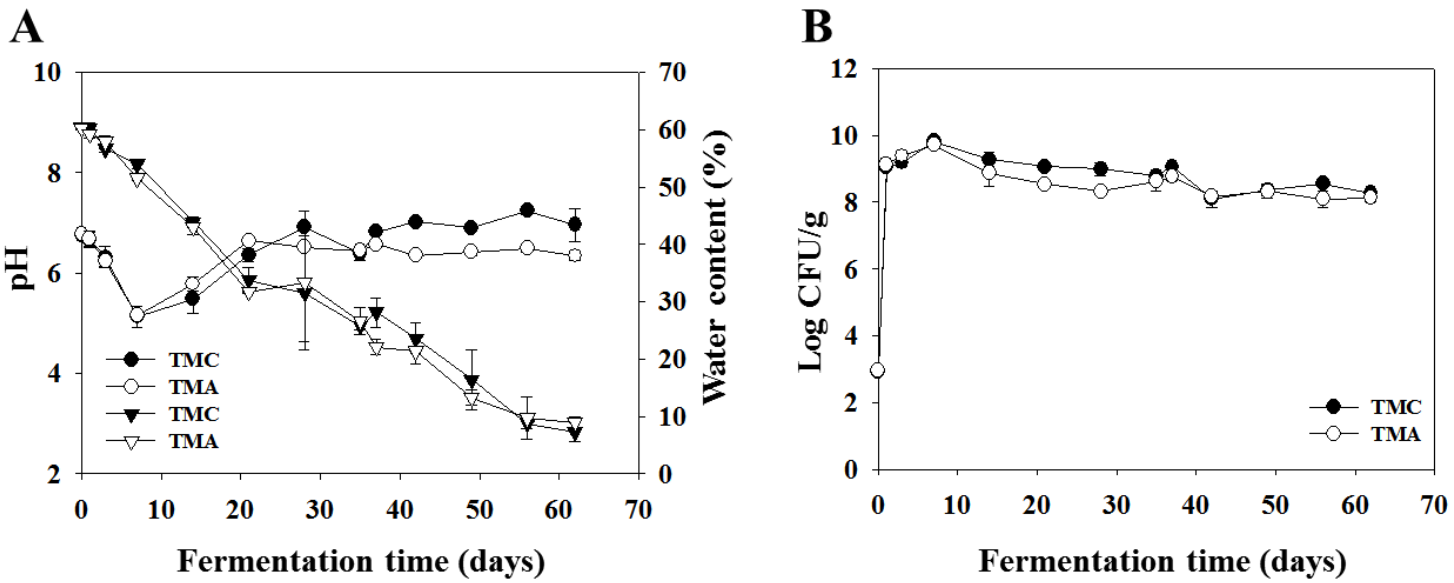

Figure IV-1. Profiles of (A) pH and water contents and (B) bacterial abundances in control and starter added meju during meju fermentation. The values were derived from triplicate meju samples and error bars represent standard deviations.

IV-3-2. Bacterial and fungal abundance during meju fermentatio

Bacterial viable cells in meju were counted on TSA agar media (**Fig. IV-1B**). The initial bacterial cells were approximately 10^3 CFU/g-fresh weight in both meju samples. After 1 day, drying period at 20℃ for 20 h, bacterial abundance rapidly increased to approximately 1.5 x 10^9 CFU/g, and 1.3 x 10^9 CFU/g-fresh weight in the TMC and TMA, respectively. The bacterial cells of both samples were reached to the highest peak of approximately 6.1 x 10^9 CFU/g, and 4.3 x 10^9 CFU/g-fresh weight at 7 day in the TMC and TMA and then it steadily decreased until the end of fermentation (62 days). While the decrease rate of bacterial abundance in TMA is a little bit faster than TMC during the middle period of fermentation (14-35 days), the bacterial abundance finally became similar at approximately 1.5 x 10^9 CFU/g in both meju samples.

A qRT-PCR approach based on the 16S rRNA gene copies was used to enumerate the total number of fungi in two meju samples during meju fermentation. The exact determination of bacterial cell numbers using qRT-PCR is almost impossible because the copy numbers of chromosomal 16S rRNA gene operons vary with species type (Farrelly, et al. 1995, Park, et al. 2009); however, qRT-PCR analysis allows for the estimation of the changes in the cell numbers of a microbial community (Jung, et al.

2014) In this study, the total numbers of fungi in the meju samples were estimated using a standard curve (TMC, R2=0.985; TMA, R2=0.986) generated from the cloned 16S rRNA gene of *A. oryzae* SNU-HR.

IV-3-3. Changes in amylase and protease activities meju fermentation

The changes of amylase and protease activities in meju samples were measured during the fermentation process. Enzyme activities were visualized as clear zone around the paper disc on the plate at the end of the incubation period. The diameter of clear zone of amylase was the almost same at initial fermentation day (0 day) in both meju samples. In TMC, the amylase activity was considerably increased until 7 day, after that it was gradually increased to reach approximately 18 mm at the end of fermentation (62 day). Compared with TMA sample, the amylase activity of TMC was very sharply increased to 22 mm, which is higher than the max value of TMC, and remained relatively constant until 62 day. Although the profile of protease activity show the tendency to steadily increased starting from early fermentation period, the protease activity in TMA was increased faster than that in TMC. The highest protease activities observed in TMC and TMA were 14 mm (62 day) and 15mm (49 day), respectively.

In conclusion, TMA sample added *A. oryzae* SNU-HR showed high value in both amylase and protease activity.

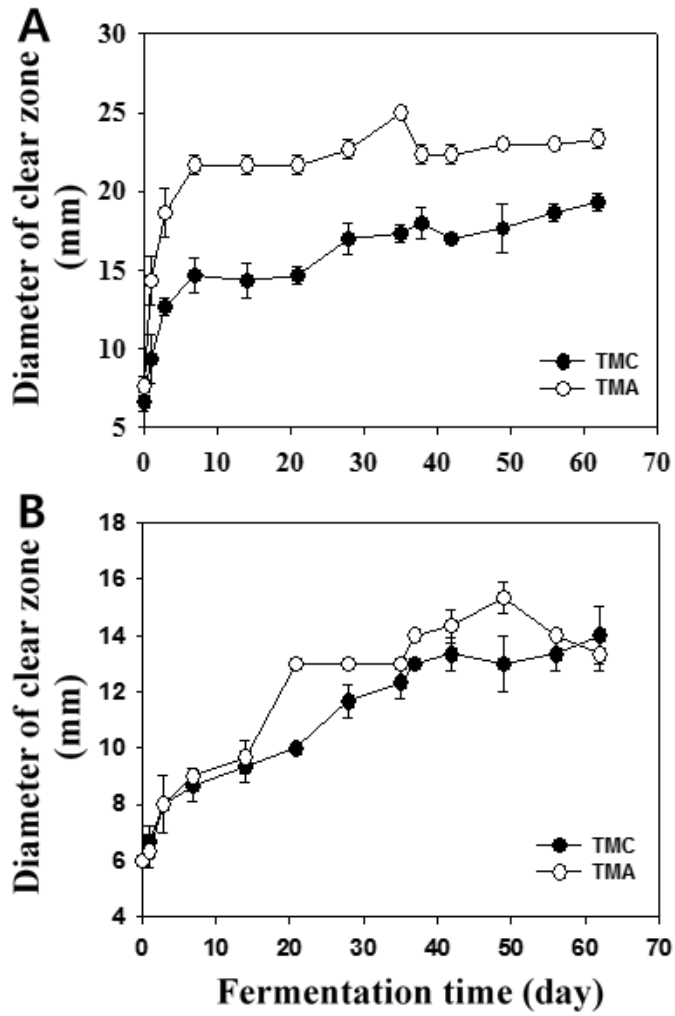

Figure IV-2. Profiles of (A) amylase and (B) protease activities in control and starter added meju during meju fermentation. The values were derived from triplicate meju samples and error bars represent standard deviations.

IV-3-4. Changes in bacterial and fungal diversity during the meju fermentation

A massively parallel barcoded pyrosequencing approach was applied for the analysis of microbial community succession during meju fermentation. Enough bacterial 16S rRNA and fungal 28S rRNA gene amplicons for TMC and TMA samples were successfully obtained from the two 1/8 regions of the PicoTiterPlate using GS FLX Titanium chemistry on a 454 Genome sequence. 306,363 sequencing reads from a single run with 48 bacterial and fungal PCR amplicons were generated. After cleaning the low quality and chimera sequences, 43,628 bacterial and 83,242 fungal high quality sequences with an average read length of approximately 2,440 reads per sample were obtained for the analysis of bacterial diversity and community. To analyze the community richness in meju samples, I calculated rarefaction curves at 97% similarity levels (Figure IV-3). All calculated diversity indices and richness estimators (OTU, Shannon–Weaver, Chao1, and evenness) supported the results of the rarefaction curve analysis, although the number of reads obtained affected the bacterial diversity indices (Table IV-2). The plots indicating the relationship between Shannon-Weaver indices, indicating species abundance and evenness present in samples, and sample type explicitly demonstrated that bacterial diversities in TMC were higher than that in TMA (Figure IV-4). Compare with the bacterial diversities,

the fungal diversity showed large discrepancy in Shannon-weaver index (H') of two meju samples. The fungal diversity in TMA is much lower than that in TMC. However, each Shannon-weaver index (H') of meju samples was fluctuated within a small range from beginning to the end of the fermentation.

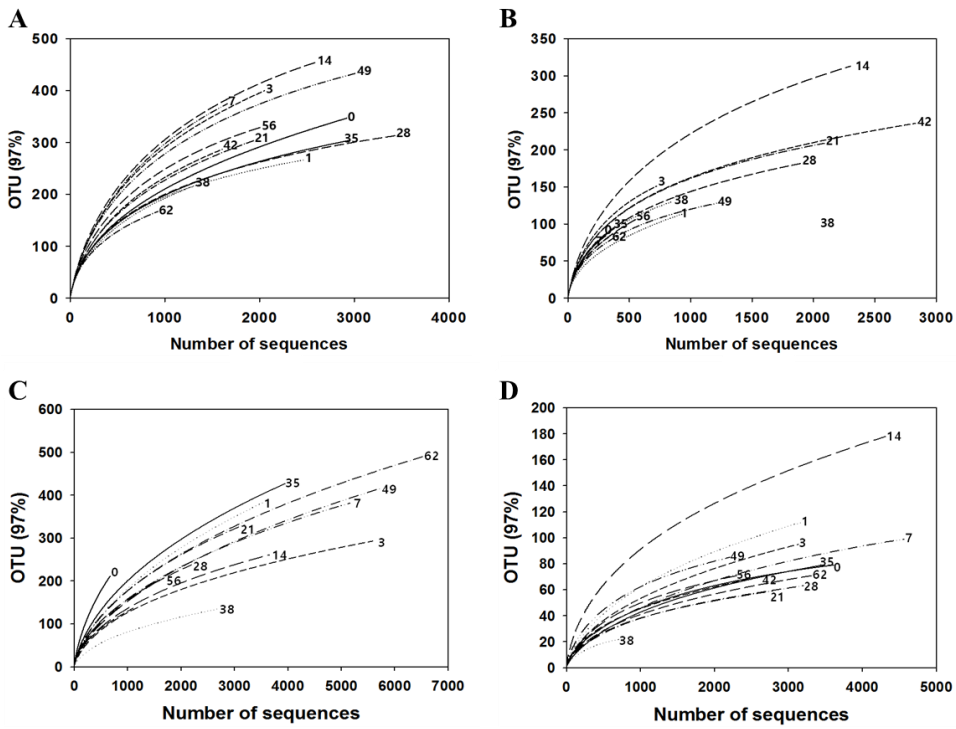

Figure IV-3. Rarefaction analysis of bacterial 16S rRNA gene sequence for the analysis of (A) TMC, (B) TMA and fungal 28S rRNA gene sequence for the analysis of (C) TMC, (D) TMA meju samples during the entire meju fermentation process. The rarefaction analysis was carried out within the RDP program with a 97 % OTU cutoff value and numbers beside the curves represent the fermentation time (in days) of the meju samples.

Table IV-2. Summary of the sequencing data sets and statistical analysis of meju samples

Sample ID (day)			Total reads	High quality	Original				Normalization			
					OTUs	Chao 1	Shannon	Evenness	OTUs	Chao 1	Shannon	Evenness
Bacteria	TMC	1	9031	2487	314	383.77	4.68	0.81	53	74	3.4	0.86
		3	6576	2054	304	417.42	4.64	0.81	53	72.13	3.47	0.87
		7	5352	1664	215	306.14	4.45	0.83	49	103.17	3.15	0.81
		14	10738	2586	167	240.97	4.40	0.86	58	72	3.49	0.86
		21	5555	1945	267	359.25	4.55	0.81	77	129.93	3.75	0.86
		28	9180	3467	400	662.89	5.16	0.86	76	142.23	3.67	0.85
		35	9634	2948	375	530.00	5.15	0.87	92	171.69	4.1	0.91
		38	3689	1294	454	571.81	5.32	0.87	68	131	3.54	0.84
		42	5636	1609	347	646.07	4.66	0.80	59	73.44	3.39	0.83
		49	7850	3010	287	417.02	4.72	0.83	74	107.07	3.84	0.89
		56	5547	2006	329	426.84	4.94	0.85	51	64.91	3.38	0.86
		62	2002	929	433	564.86	5.16	0.85	69	94.07	3.76	0.89
	TMA	1	2420	942	182	254.33	4.14	0.80	40	60.00	3.12	0.85
		3	2784	724	94	141.30	3.88	0.85	39	56.50	2.97	0.81
		7	772	241	130	191.89	4.03	0.83	45	72.14	3.18	0.83
		14	6729	2303	86	138.93	3.84	0.86	53	95.86	3.38	0.85
		21	4932	2089	114	200.13	3.59	0.76	54	71.10	3.50	0.88
		28	6528	1906	151	197.67	4.31	0.86	54	97.88	3.20	0.80
		35	753	377	78	148.71	3.69	0.85	72	101.18	3.73	0.87
		38	1767	847	313	434.39	4.74	0.82	56	71.00	3.51	0.87
		42	9505	2835	84	125.63	3.79	0.85	38	49.00	2.72	0.75
		49	4486	1215	236	343.25	4.33	0.79	42	55.33	3.00	0.80
		56	1397	551	106	174.06	3.80	0.82	40	46.88	3.11	0.84
		62	770	366	128	165.27	3.83	0.79	39	57.20	3.00	0.82

Fungi	TMC	1	5193	3556	384	734.58	3.88	0.65	128	418.50	3.39	0.70
		3	7738	5594	293	463.30	2.78	0.49	81	222.43	2.39	0.54
		7	7552	5167	382	653.73	3.59	0.60	89	171.33	3.17	0.71
		14	7457	3655	261	447.15	3.18	0.57	79	134.71	2.60	0.60
		21	5039	3194	332	605.90	3.89	0.67	120	195.12	3.34	0.70
		28	3862	2202	238	437.49	3.68	0.67	101	189.40	3.12	0.68
		35	6715	3948	427	778.04	4.04	0.67	122	203.38	3.36	0.70
		38	997	737	22	24.63	0.64	0.21	30	43.13	0.69	0.20
		42	1650	1108	137	305.81	3.34	0.68	75	110.77	2.70	0.62
		49	8933	5765	417	776.55	3.69	0.61	103	233.71	3.13	0.68
		56	2991	1681	209	459.00	3.42	0.64	88	172.00	2.72	0.61
		62	13182	6558	491	889.53	3.67	0.59	104	176.53	2.86	0.62
	TMA	1	5975	3177	112	196.18	1.64	0.35	58	132.00	1.71	0.42
		3	7577	3133	95	156.88	1.18	0.26	63	137.55	1.34	0.32
		7	9578	4569	99	127.89	1.00	0.22	51	113.33	1.10	0.28
		14	8493	4318	178	290.54	2.39	0.46	70	128.67	2.30	0.54
		21	6992	2788	59	89.67	0.89	0.22	49	95.50	1.14	0.29
		28	8897	3334	71	104.46	1.09	0.25	39	54.30	1.15	0.31
		35	7229	3469	79	116.40	0.78	0.18	41	68.60	0.93	0.25
		38	1580	737	22	23.67	0.72	0.23	43	89.43	0.99	0.26
		42	6597	2536	69	112.50	1.07	0.25	55	100.09	1.21	0.30
		49	5848	2209	85	131.50	1.51	0.34	62	167.86	1.66	0.40
		56	6612	2311	71	121.75	0.95	0.22	60	112.50	1.24	0.30
		62	10497	3201	63	103.63	0.82	0.20	44	50.32	1.10	0.29

OTUs, operational taxonomic units. Diversity indices of bacterial and fungal communities in each sample were calculated using the RDPipeline based on the 16S rRNA gene sequences at a 97% cutoff value.

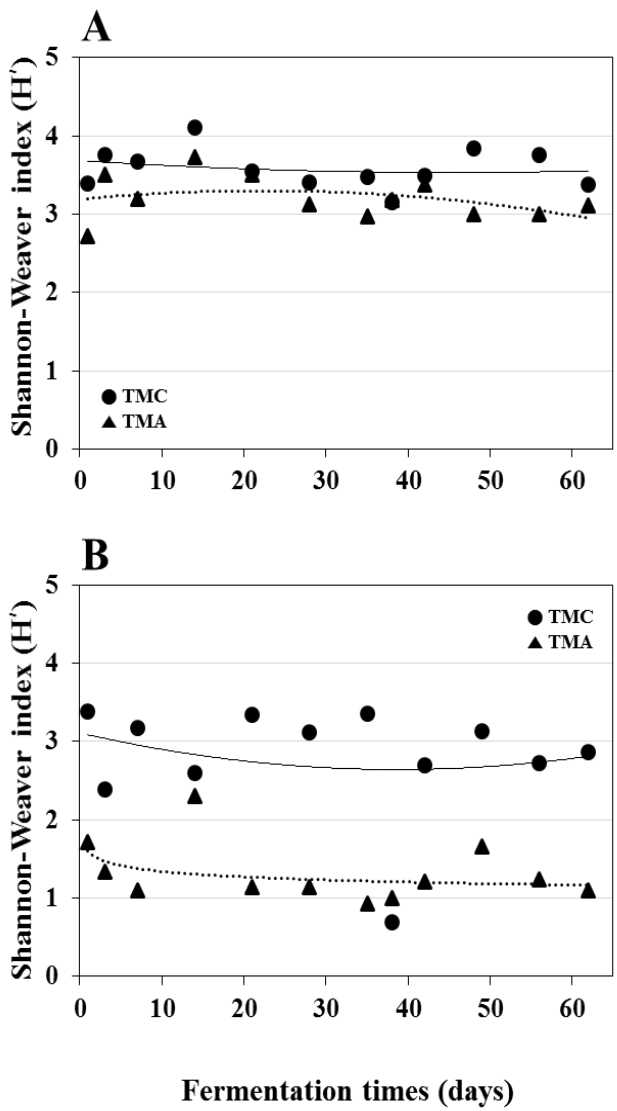

Figure IV-4. Plots showing the relationships between Shannon-Weaver indices of (A) bacteria and (B) fungi and the fermentation times (in days) during the meju fermentation.

IV-3-5. Changes in bacterial and fungal communities during the meju fermentation

The high quality pyrosequencing reads of bacterial 16S and fungal 28S rRNA gene sequences were classified at both the phylum and genus levels in order to investigate the microbial community changes during the meju fermentation. The phylum-level analysis for bacteria revealed that only *Firmicutes* predominated **Figure IV-5.** A and B, which is consistent with previous reported results for other fermented meju (Jung, et al. 2014). *Proteobacteria* was also detected in both samples during the whole fermentation day as a minor group in the range of 0.08% to 5.6 %.

The genus-level of bacteria analysis revealed *Bacillus*, unclassified Bacillales, *Staphylococcus*, *Weissella*, unclassified Bacilli, unclassified Bacillaceae, *Leuconostoc*, *Lactococcus* which might be primarily derived from raw materials, were identified in both initial meju samples (day 0), although their relative abundance showed a slight differences (**Figure IV-5.** C and D).

At the genus level of bacteria, *Bacillus* and unclassified Bacillaceae belonging to the phylum *Firmicutes* were predominant in both meju samples, during the entire fermentation period. While the growth of the genera *Bacillus* and unclassified Bacillaceae showed relatively the highest abundance between 21 days and 35 days

of fermentation and their relative abundance fell slightly in the latter in TMC, however, the growth of these two bacteria was rapidly increased and became dominant at 21 day in TMA and its high relative abundance lasted until the end of fermentation (62 day). Genera *Staphylococcus*, considerably decreased until 21 day and then gradually increased again, became relatively predominant after 35 day in TMC. However, the genera *Staphylococcus*, its relative abundance was approximately 57%, was almost disappeared after 1 day. Considerable growth of the genera *Weissella*, uncultured *bacilli*, and *Leuconostoc* also observed in both samples during the early fermentation period (14 day), but the genera was significantly declined after 14 day and 7 day in TMC and TMA, respectively. The relative abundance of *Weissella* in TMC was higher than TMA, by contrast, the relative abundance of *Leuconostoc* was much higher in TMA. Unclassified *Thermoactinomycetaceae* 1 was newly appeared after 38 days of fermentation in TMC. *Clostridium sensu stricto* was continually identified and their abundance were nearly constant in both meju samples from 7 days to the end of fermentation. The growth of diverse bacterial groups such as *Sporolactobacillus*, *Acinetobacter*, and *Brochothrix* were also observed.

Interestingly, the level of *Staphylococcus*, some strains have been used as a starter for fermented food but mostly recognized as a pathogen, was much higher in TMC than that in TMA. The relatively abundance of *Staphylococcus* was about 35% at 1 day, which decreased and sustain about less than 20 % with small-fluctuation until 35 day, it suddenly increased again to the base level at 38 day and maintained that level until 62 day.

At the phylum level of fungi, *Ascomycota* and *Fungi incertae sedis* identified as the predominant phyla during the entire meju fermentation in TMC, whereas *Ascomycota* was absolutely predominant in TMA (**Figure IV**-6. A and B). The phylum *Ascomycota* was predominant during the whole fermentation period, but its relative abundance suddenly decreased at 7 and 21 day in TMC. *Fungi incertae sedis* was observed about 15-20 % during fermentation period except for 7, 21 day, which was opposite to the composition of Ascomycota.

The genus-level of fungi analysis revealed very diverse fungal flora, which derived from raw material, water, air, and jip, are existed in TMC. However, the fungal flora of TMA added *A. oryzae* SNU-HR as starter was much simple during the whole fermentation period (**Figure IV**-6. C and D). At the genus level of the fungal 28S rRNA gene sequences showed that the genera *Aspergillus*, unclassified

Microascaceae, *Gibberella*, and *Mucor* primarily represented the fungal communities during entire fermentation day in TMC. However, *Hanseiaspora* and *Sordaria* were only observed with a large percentage at 3 and 14 day, respectively. The genus *Mucor* belonging to the phylum *Fungi incertae sedis* dominated at 7 days (75.1%), and then deceased and members of other genera such as *Aspergillus* and unclassified *Microascaceae* increased and became dominant during the late fermentation period. The genus *Gibberella*, detected with small percentage at 1 day, rapidly increased at 21 day and constantly detected until the end of the fermentation. In TMA, although *Hanseniaspora*, *Mucor*, *Sordaria* were rarely detected during fermentation period, the genus *Aspergillus* was absolutely predominant during whole fermentation period, suggesting that *A. oryzae* SNU-HR, added as meju starter, was successfully settled and it probably play key roles in meju fermentation. Other minor fungal genera detected in the meju samples included *Cladosporium complex*, *Stagonosporopsis*, *Penicillium*, *Nigrospora*, *Doratomyces*, *Christansenia*, *Guehomyces* and various unclassified fungal genus; these groups did not exhibit dramatic fluctuations in their relative abundance during fermentation.

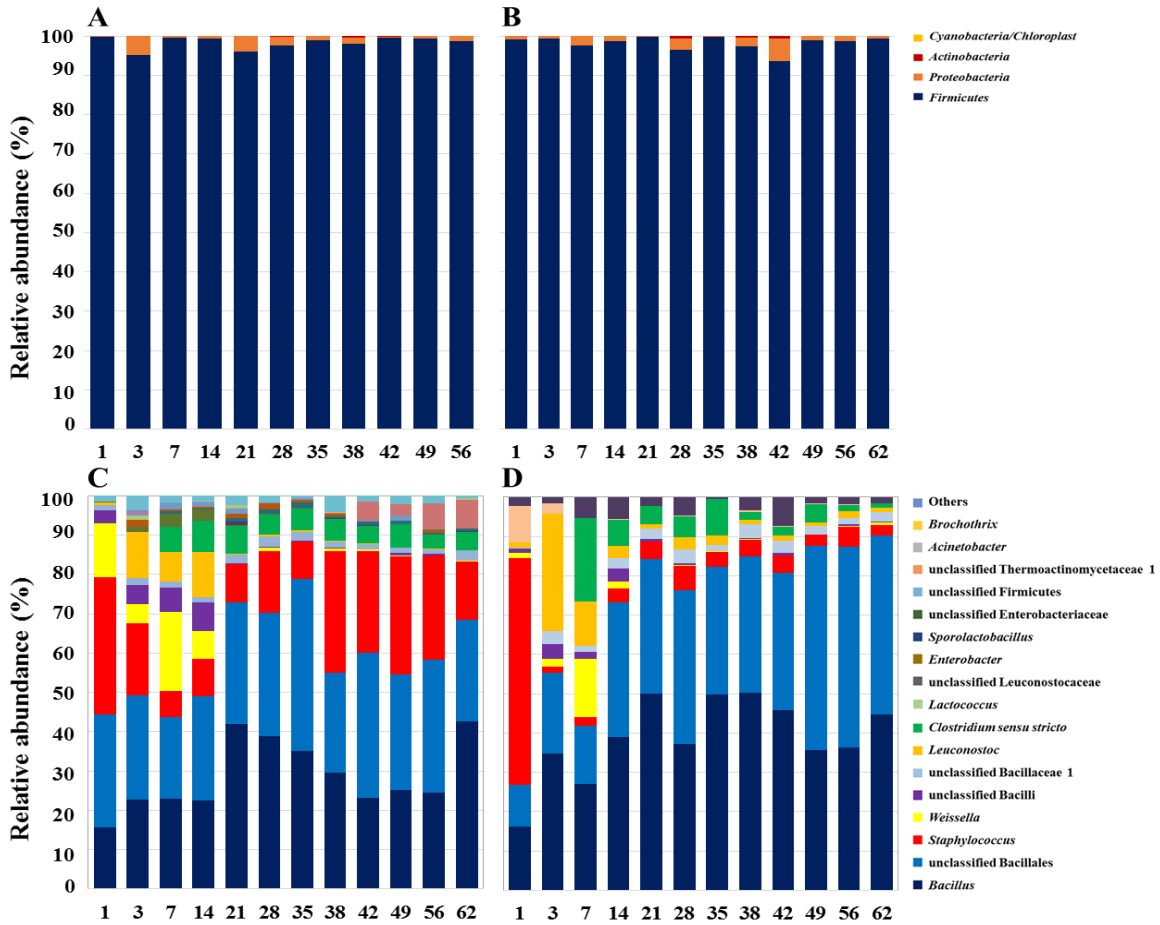
A
B
C
D
Relative abundance (%)
100
90
80
70
60
50
40
30
20
10
0
1 3 7 14 21 28 35 38 42 49 56
1 3 7 14 21 28 35 38 42 49 56 62
Cyanobacteria/Chloroplast
Actinobacteria
Proteobacteria
Firmicutes
Others
Brochothrix
Acinetobacter
unclassified Thermoactinomycetaceae 1
unclassified Firmicutes
unclassified Enterobacteriaceae
Sporolactobacillus
Enterobacter
unclassified Leuconostocaceae
Lactococcus
Clostridium sensu stricto
Leuconostoc
unclassified Bacillaceae 1
unclassified Bacilli
Weissella
Staphylococcus
unclassified Bacillales
Bacillus

Figure IV-5. Bacterial community changes in the control (A and C) and *A. oryzae* SNU-HR (B and D) added mejus during the meju fermentation. Data portray phylum (A and B) and genus (C and D) level analyses of bacterial 16S rRNA gene sequences derived from the meju samples. The 16s rRNA gene sequences with more than 300 bp were classified using the RDP naïve Bayesian rRNA Classifier at an 80% confidence threshold. The 'others' in panels C and D are composed of the genera, each showing a percentage of reads <1.0% of the total reads in all samples.

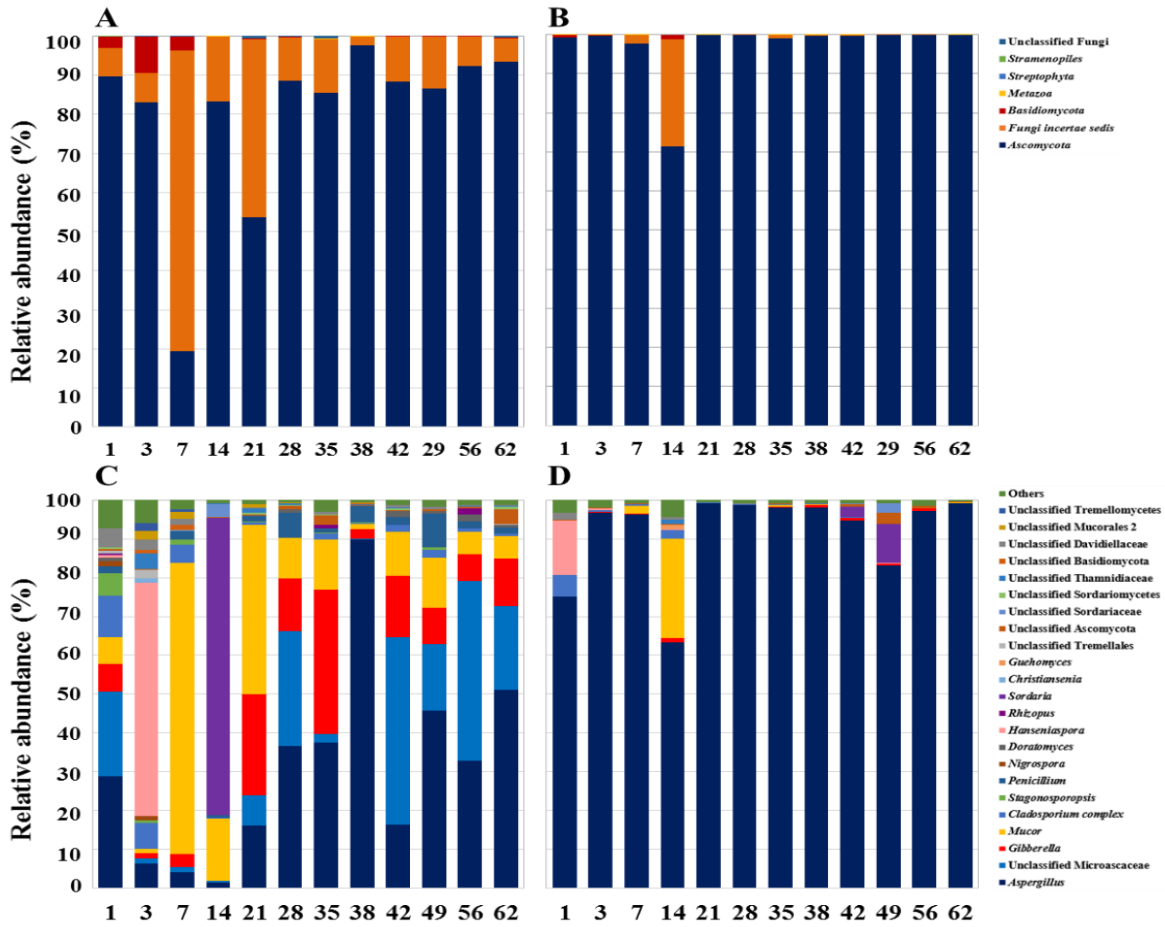
A
B
C
D
Relative abundance (%)
100
90
80
70
60
50
40
30
20
10
0
1 3 7 14 21 28 35 38 42 29 56 62
1 3 7 14 21 28 35 38 42 49 56 62
Unclassified Fungi
Stramenopiles
Streptophyta
Metazoa
Basidiomycota
Fungi incertae sedis
Ascomycota
Others
Unclassified Tremellomycetes
Unclassified Mucorales 2
Unclassified Davidiellaceae
Unclassified Basidiomycota
Unclassified Thamnidiaceae
Unclassified Sordariomycetes
Unclassified Sordariaceae
Unclassified Ascomycota
Unclassified Tremellales
Guehomyces
Christiansenia
Sordaria
Rhizopus
Hanseniaspora
Doratomyces
Nigrospora
Penicillium
Stagonosporopsis
Cladosporium complex
Mucor
Gibberella
Unclassified Microascaceae
Aspergillus

Figure IV-6. Fungal community changes in the control (A and C) and A. oryzae SNU-HR (B and D) added mejus during the meju fermentation. Data portray phylum (A and B) and genus (C and D) level analyses of bacterial 28S rRNA gene sequences derived from the meju samples. The 16s rRNA gene sequences with more than 300 bp were classified using the RDP naïve Bayesian rRNA Classifier at an 80% confidence threshold. The 'others' in panels C and D are composed of the genera, each showing a percentage of reads <1.0% of the total reads in all samples.

IV-3-6. Multivarite statistical analysis

The microbial community successions of the meju samples were also compared by canonical redundancy analysis (RDA) based on the relative abundance of taxonomical community structures (Figure IV-7). RDA showed that the bacterial communities were separately grouped between the TMC and TMA regardless of the meju fermentation period except for initial sample (1, 3, and 7 day) of TMA. The statistical bacterial RDA plot analysis clearly showed that *Staphylococcus* was the most influential group in the bacterial communities of most TMC and only early TMA sample (1 day) and *Bacillus*, unclassified Bacillales became the dominant bacterial groups in both meju samples.

Similarly, the fungal RDA plot was also clearly divided into two group by meju sample types and the plot indicated that *Aspergillus* was the most influential group in the fungal communities during the entire fermentation period in TMA, whereas, the relative abundance of *Aspergillus* and. Unclassified Microascaceae was steadily increased then became dominant after 14 day in TMC.

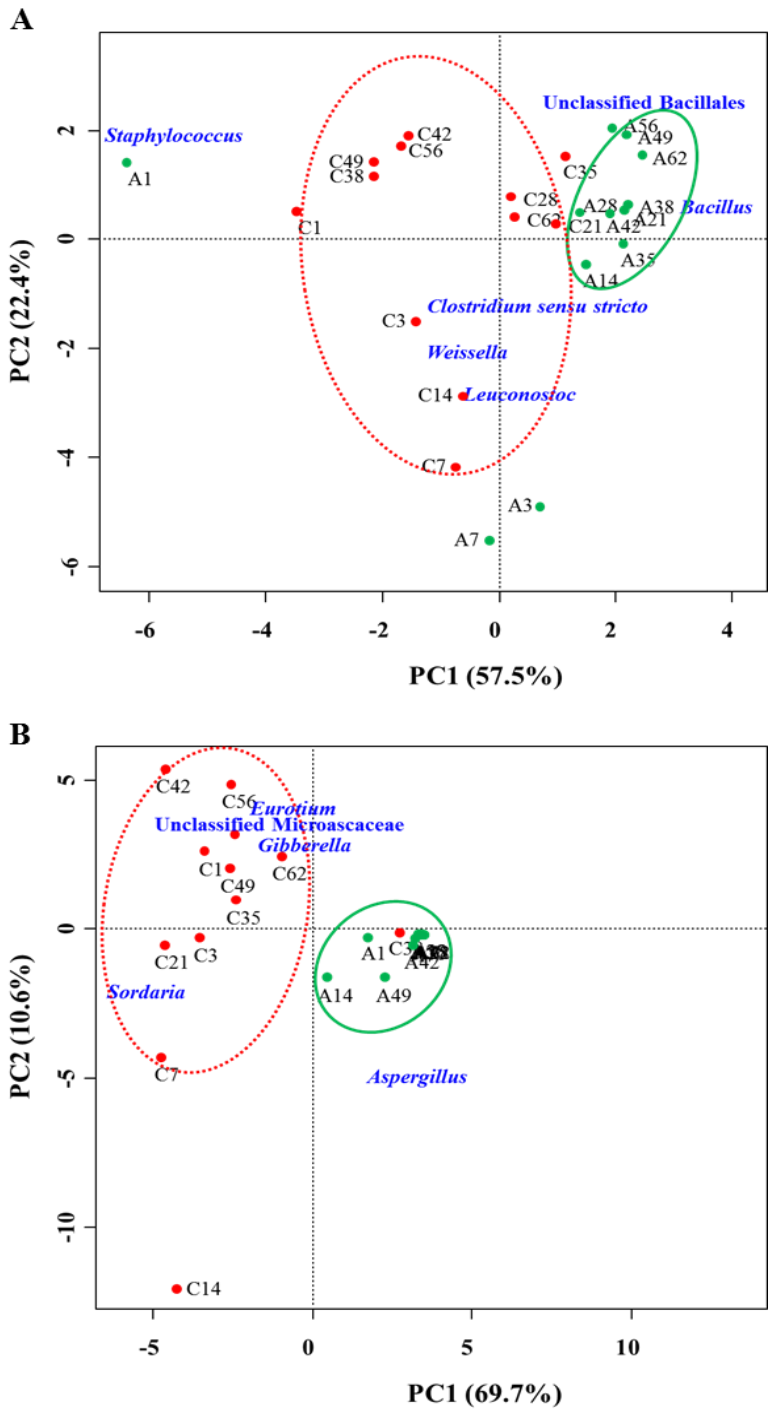
A
Staphylococcus
A1
C42
C56
C49
C38
C1
Unclassified Bacillales
A56
A49
A62
C35
C28
C62
A28
A38
Bacillus
C21
A42
A21
A35
A14
Clostridium sensu stricto
C3
Weissella
C14
Leuconostoc
C7
A3
A7
PC2 (22.4%)
PC1 (57.5%)
B
C42
C56
Eurotium
Unclassified Microascaceae
Gibberella
C1
C62
C49
C35
C21
C3
Sordaria
C7
A1
A14
A49
Aspergillus
C14
PC2 (10.6%)
PC1 (69.7%)

Figure IV-7. Score plots of the the canonical redundancy analysis (RDA) derived from the relative abundance of (A) bacterial and (B) fungal communities during meju fermentation. Numbers bside the symbols indicate the fermentation time (in days) of the meju sample. The dotted straight arrows represent the relative loadings of the taxonomic genus groups and this lengths are proportional to their influence on the microbial communities. The directions of the curved arrows in panel B indicate the routes of fungal community changes in the TMC (dotted line) and TMA (straight line) regions during the meju fermentation.

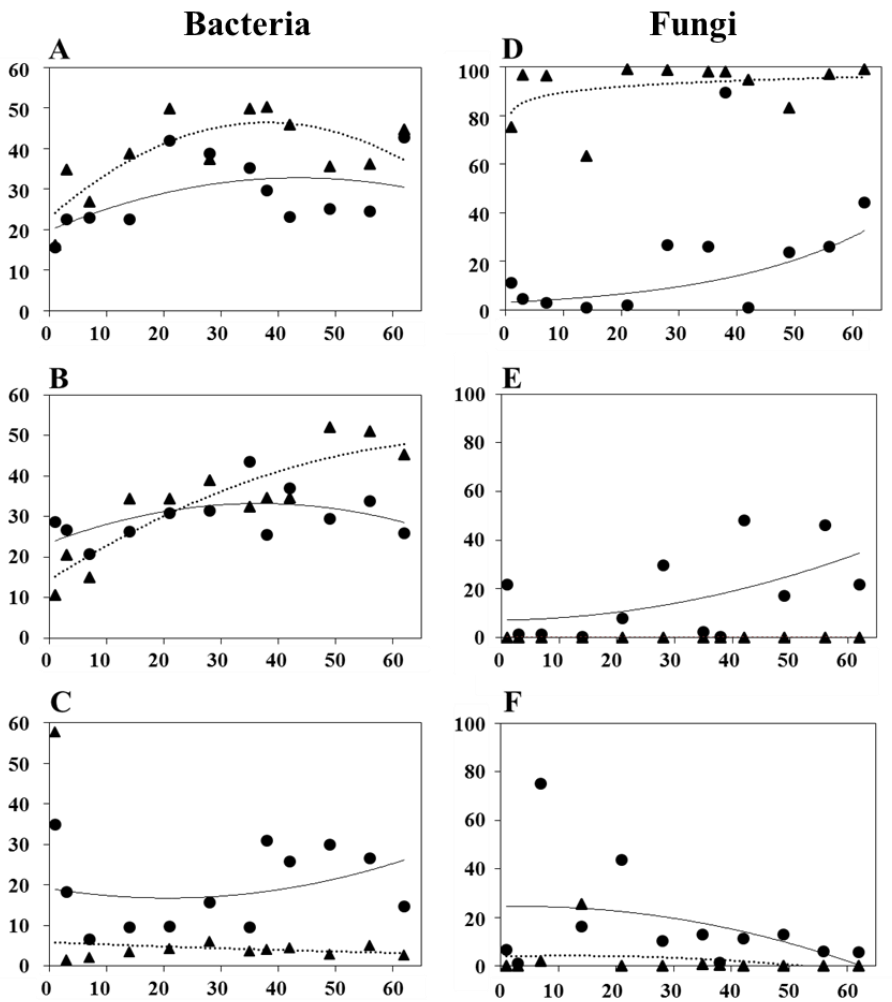

Figure IV-8. Community plots showing the relative abundance changes of predominant species in TMC (circle) and TMA (triangle). A, *Bacillus*; B, *unclassified Bacilliceae*; C, *Staphylococcus*; D, *Aspergillus*; E; *unclassified Microascaceae*; F, *Gribberella*.

Table IV-3. Pearson's correlation coefficients between characteristics of meju (pH, water contents (%), amylase, protease) and bacterial and fungal community composition (%) during the meju fermentation period

Genus		Pearson's correlation coefficients			
		pH	Water content	amylase	protease
Bacteria	Bacillus	.105	-.504*	.740**	.606**
	Unclassified Bacillales	.275	-.691**	.487*	.712**
	Staphylococcus	.477*	.214	-.611**	-.350
	Weissella	-.695**	.628**	-.447*	-.609**
	Unclassified Bacilli	-.679**	.678**	-.516**	-.677**
	Unclassified Bacillaceae1	.018	-.222	.508*	.396
	Leuconostoc	-.480*	.573**	-.124	-.515*
	Clostridium sensu stricto	-.523**	.062	.237	.002
	Lactococcus	.129	.404	-.249	-.457*
	Enterobacter	-.078	-.237	.323	.331
	Sporolactobacillus	.137	-.248	-.066	.220
Fungi	Aspergillus	-.004	-.208	.797**	.350
	Cladosporium complex	-.029	.614**	-.745**	-.685**
	Eurotium	.425*	-.102	-.513*	-.039
	Gibberella	.244	-.171	-.339	.063
	Hanseniaspora	-.010	.375	-.376	-.378
	Mucor	-.479*	.254	-.382	-.305
	Nigrospora	.056	.553**	-.652**	-.589**
	Penicillium	.339	-.161	-.308	.092
	Rhizopus	.307	-.249	-.080	.120
	Stagonosporopsis	.013	.412*	-.591**	-.456*
	Unclassified Davidiellaceae	.009	.581**	-.730**	-.646**
	Unclassified Microascaceae	.575**	-.272	-.294	.145

IV-3-7. Metabolite changes during the meju fermentation

Analysis of metabolites, including free sugars, organic acids, and amino acids compounds, in meju was carried out using ^{1}H NMR during the entire fermentation. The metabolite analysis showed that fructose, fructose, galactose, and sucrose were identified as the major free sugar compounds from the meju samples and the free sugar levels increased quickly during the early fermentation period (Figure IV-9). However, after approximately 7-14 days, the concentrations of fructose and galactose decreased relatively quickly and they were consumed almost completely at the end of fermentation. However, the level of glucose rapidly declined after 7 day, and then the level sustained, and its level dropped to the basic level (approximately 20 μmol/g). The sucrose level, which was initially high (150 μmol/g), very quickly decreased to almost zero within 14 days. The level of free sugar were always higher in TMA than TMC.

Acetate, butyrate, and lactate were identified as major organic acids during the meju fermentation (Figure IV-10), the level of which increased quickly during early fermentation days, but the rapid decrease of these level were observed after 21 day. Citrate and propionate were also identified as minor organic acids during the meju fermentation. The level of citrate was the maximum at 1 day and it rapidly dropped

at 3 day. The level of propionate gradually increased until 28 day in TMC, whereas its level steadily decreased until the end of fermentation after reaching the maximum level at 7 day in TMA.

It is well kwon that the proteolysis of proteins to amino acids in fermented food is an important process that enhances the flavor and taste of fermented soybean products. The concentrations of amino acids rapidly increased during the early fermentation period, but their concentration (i.e., alanine, arginine, asparagine, aspartate, glutamate, glutamine glycine, and valine) gradually decreased after 42 day as the fermentation processed (Figure IV-11). Interestingly, the concentration of most amino acids including alanine, asparagine, aspartate, glutamate, glutamine, and glycine were much higher in TMA.

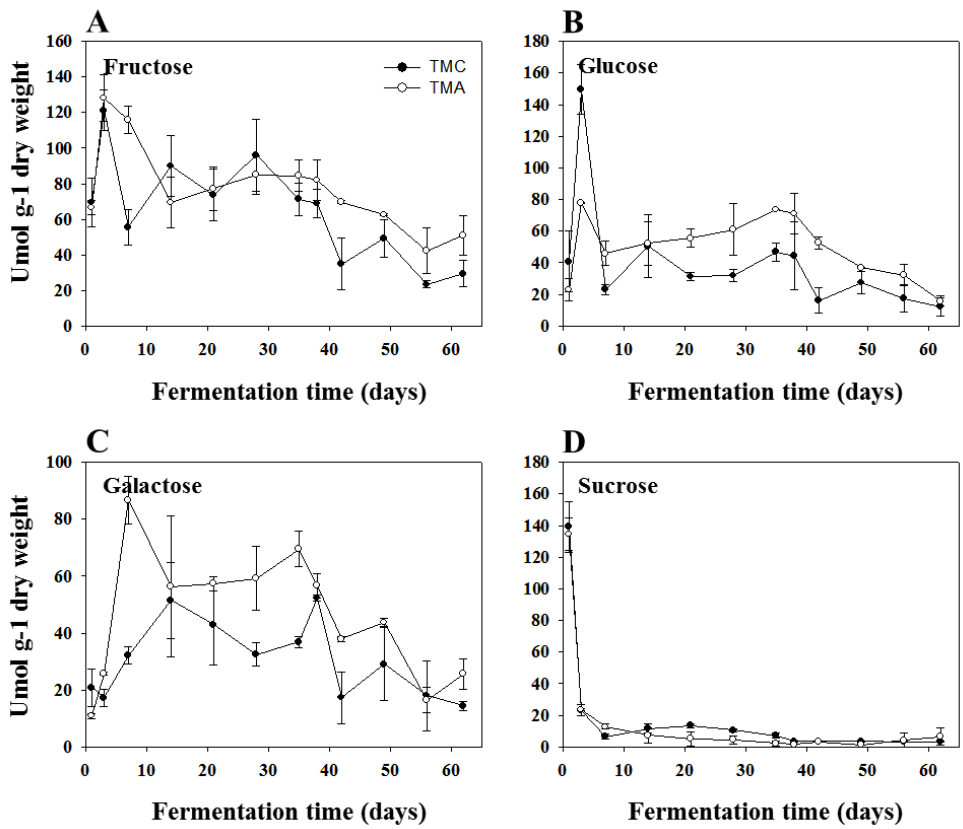

Figure IV-9. Changes of carbohydrates between TMC and TMA samples during the meju fermentation. Data are given as means ± standard errors.

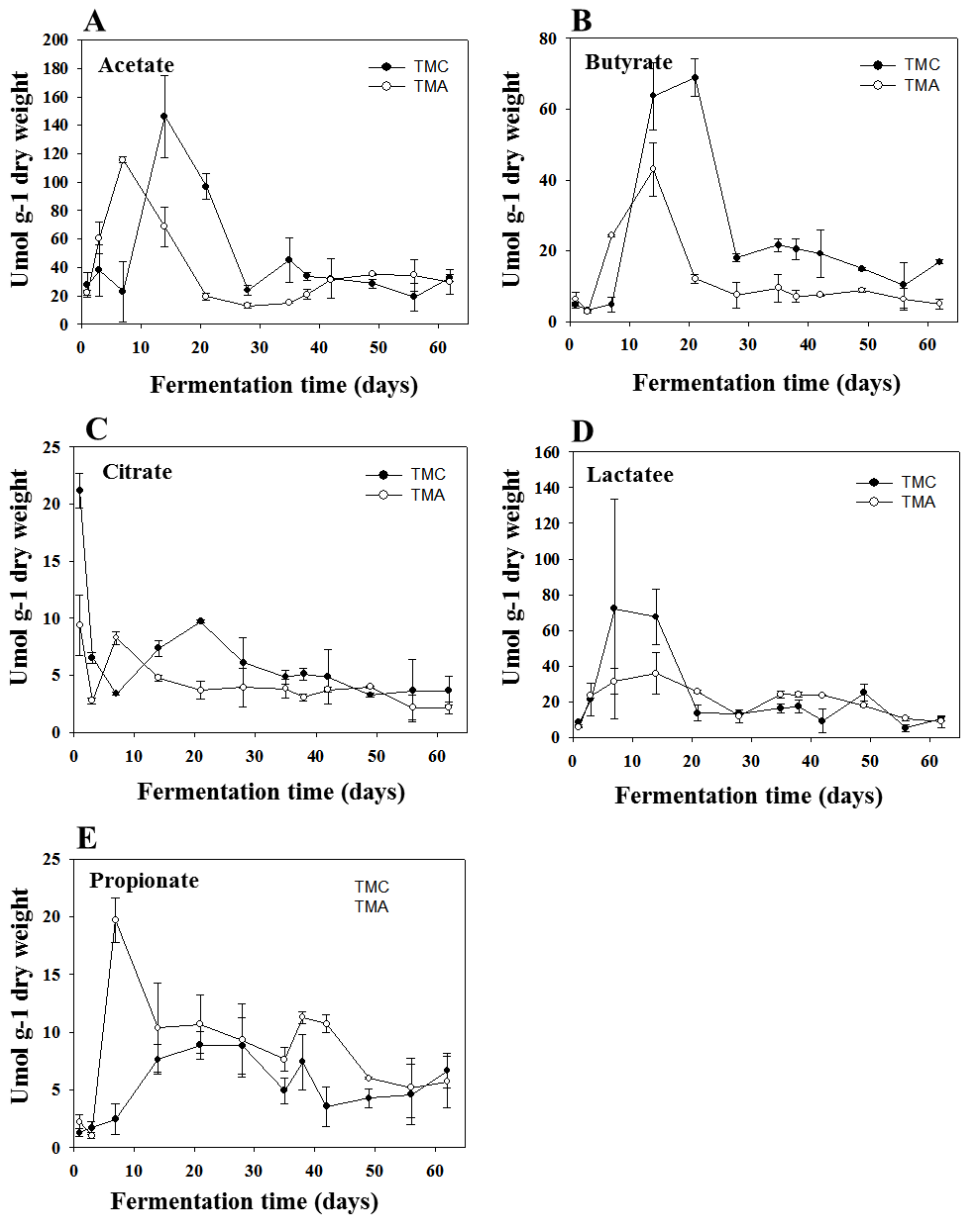

Figure IV-10. Changes of major organic acids between TMC and TMA samples during the meju fermentation. Data are given as means ± standard errors.

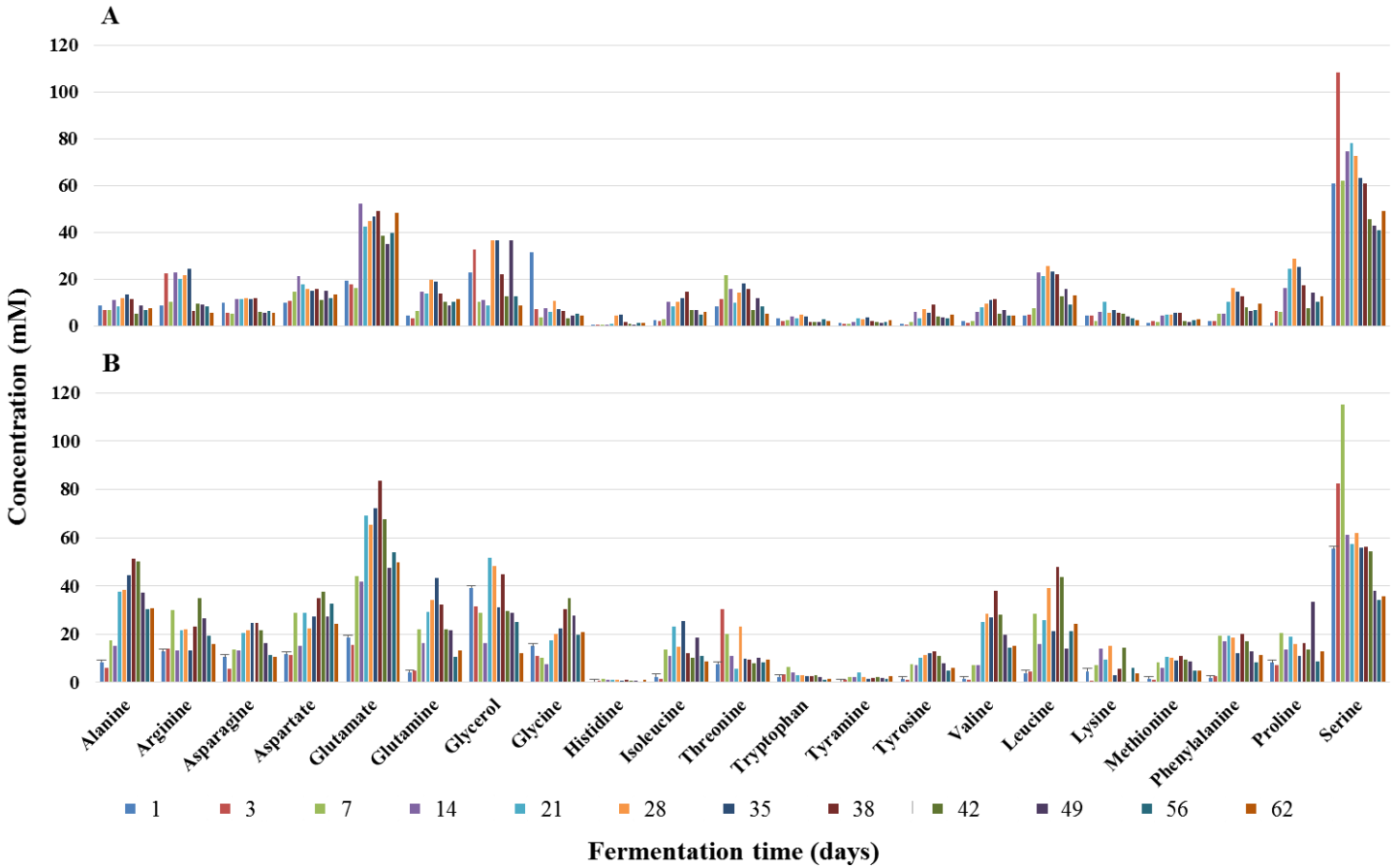

Figure IV-11. Changes of amino acids between TMC (A) and TMA (B) samples during the meju fermentation.

IV-4. Discussion

Korean traditional fermented soybean product, doenjang, has been recognized as a very important food source for seasoning and occupies major portion of the diet in Korea (Mo, et al. 2010, Yang, et al. 2011). The consumption of doenjang has been gradually increased because of its excellent nutritional value and beneficial effect on human health such as anticancer activity, antimutagenic activity, and high blood pressure depressant ability (Shim, et al. 2015, Park, et al. 2003, Shin, et al. 2015). Moreover, previous study reported that the consumption of doenjang helps not only enhance immune function but also prevent allergies (Kwak, et al. 2015). Although doenjang have so many advantages, it sometimes threatens human health by producing toxic substances such as mycotoxin, aflatoxin, and BAs (Park, et al. 2003, Shukla, et al. 2010).

The doenjang quality changes depending on various factor such as ambient temperature, salt, and ingredients. Among them, the fermented soybean brick (meju), which is the main ingredient for manufacturing doenjang in Korea, is probably the biggest influence to doenjang quality as meju represents very complex microbial ecosystem comprising bacteria as well as fungi derived from jip, ambient air, and

worker. The conditions of traditional meju fermentation are so various that it is difficult to standardize and unify quality and make it impracticable to commercialize. Consequently, the meju quality need to control to overcome inconsistent quality and become feasible commercialize of doenjang, guaranteed by high quality and safety. Starter cultures cause changes to the microbial constitution of meju, speed up the produce of metabolites, flavor, aroma, and in general bring about the formation of the characteristic qualities of the product.

In this study, I prepared meju samples by Korean traditional method and added *A. oryzae* SNU-HR, which is aflatoxin- as well as CPA-non-producer strain, isolated from industrial koji and a pyrosequencing approach and ^{1}H-NMR strategy were applied to investigate microbial communities and metabolites in meju, respectively. It is well known that various metabolites such as amino acid, organic acid are produced by extracellular enzymes, generated by microorganisms, and it affects the organoleptic taste, flavors, and functionalities of doenjang. The meju manufactured with *A. oryzae* SNU-HR (TMA) showed the higher amylase and protease activities than TMC. It was inferred that the high amylase activity, reached the maximum value at 7 day, may helped the hydrolysis of soybean, which might be supported by the fact that most metabolites including alanine, asparagine, aspartate, valine, and lysine

were produced almost 2 times more in TMA than TMC. These results show that fungi probably be more responsible for meju fermentation.

To investigate the effect of bacterial and fungal communities on enzyme activities, Pearson's correlation coefficients and P values between the compositions of bacterial and fungal community and enzyme activities during meju fermentation period were calculated. As a result, *Bacillus* and unclassified *Bacillales* had a high correlation coefficient with amylase and protease activity but all fungal taxa had a low correlation coefficients with both enzyme activities except for *Aspergillus*, which showed high correlation coefficients with amylase activity ($P < 0.05$, absolute values of Pearson's correlation coefficients of > 0.6). *Weisella*, unclassified *Bacilli*, *Leuconostoc*, and *Clostridium sensu stricto* were showed a negative correlation with pH during the meju fermentation (Table IV-3).

Here, I analyzed the changes of microbial community focused on both bacteria and fungi during the entire fermentation period and found that inoculating fungi to meju affected into bacterial community as well as fungal community. The bacterial community analysis showed that microbial composition was very similar but their relative abundance was difference in two meju samples whereas the fungal community composition, showed opposite to bacterial community, was totally

difference between TMC and TMA. The genera *Aspergillus*, unclassified *Microascaceae, Gibberella, Mucor, Cladosporium complex, Stagonosporopsis, Penicillium, Nigrospora, Hanseniaspora, Rhizopusm, Sordaria, Christiansenia*, and *Guehomyces* were detected in TMC however *Aspergillus* was overwhelmingly detected.

The bacterial community analysis showed that *Bacillus* was the predominant populations in meju during the entire fermentation period (Figure IV-5). *Bacillus* has been generally accepted that *Bacillus* is primarily responsible for meju fermentation because the genus *Bacillus* have been always observed predominantly from meju samples by culture dependent and culture independent method approach (Jeong, et al. 2014, Kim, et al. 2009). In addition, our study also showed that the abundance of *Bacillus* was relatively constant during the entire fermentation period regardless using starter.

Interestingly, the genus *Staphylococcus* significantly decreased and was replaced by *Bacillus* and unclassified *Bacillaceae* in TMA. Generally, the genus of *Staphylococcus* is recognized as the pathogens of man and other mammals and it is divided into two groups on the basis of their ability to clot blood plasma. The coagulase positive staphylococci constitute the most pathogenic species than

coagulase negative staphylococci (CNS). The genus *Staphylococcus* including *S. equorum, S. nepalensis, S. saprophyticus, S. succinus, S. vitulus, S. warneri*, and *S. xylosus* which are all CNS, has been isolated from meju as well as many fermented products, likewise the genus *Staphylococcus* is widespread in nature and common commensals of skin (Schleifer, et al. 1975), it is assumed that *Staphylococcus* were derived from the surrounding environment including ambient air and facility during the process of meju. Besides, some CNS have been used even starter for fermented products such as sausage (Olesen, et al. 2004). Despite it is natural that *Staphylococcus* was observed as a dominant group in meju and it seems that *Staphylococcus* species present in meju do not have pathogenic properties. However, their function is not clearly revealed in meju fermentation process and some CNS also can cause infections and are increasingly recognized as agents of clinically significant infection of the bloodstream. Therefore, it could make consumer reluctance to consumption of doenjang and be export obstacles. When we prepared meju using *A. oryzae* SNU-HR, the relative abundance of *Staphylococcus* was almost disappeared.

To the best of our knowledge, this was the first study to simultaneously investigate the changes of microbial communities and metabolites together in meju, which was

prepared using starter during entire fermentation. Using fungi as starter seemed that it helps to widen crack and surface areas of meju by drying and thus the relative abundance of *Bacillus*, obligate aerobic bacteria, could growth faster in TMA than TMC, supported the result of water content. Furthermore, the exogenous enzymes of *Bacillus* and *Aspergillus*, considered to play roles in meju, may reduce aging time of meju, and thus to solve economical disadvantages caused by a long period of consumption. Although I explored meju by the simultaneous analysis of microbial community and metabolites in this study and additional researches, especially by the applications of various "omics" technologies including metagenomics, metatransciptomics and metabolomics, are surely required for a better understanding of meju fermentation, which will provide insight into the production of safe and high-quality Korean traditional meju.

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국문초록

한국의 전통 콩 발효식품인 메주와 된장의 미생물 군집 및 대사체 분석과 *Bacillus subtilis* 판지놈 분석 연구

된장은 쌀을 주식으로 하고 육류 자원이 풍부하지 못한 우리 민족에게 부족되기 쉬운 단백질, 필수 아미노산 및 필수 지방산 등의 영양성분을 공급해주는 중요한 영양원 및 조미식품으로 사용되어 왔으나, 먹거리가 풍부해진 현대에는 단백질 공급 기능 외에 이들이 가지는 생체 조절 기능을 이용하기 위한 장류의 소비가 증가하는 추세에 있다. 장류 발효에는 다양한 세균 및 곰팡이가 관여하며 발효가 진행되는 동안 된장의 주 원료인 콩의 단백질, 탄수화물, 지방이 이들 미생물이 분비하는 세포외 효소에 의해 아미노산, 단당류 지방산 등으로 분해된다. 이때, 생성된 기능성 펩타이드에 의해 된장 고유의 풍미를 가지게 되며, 이외에 항암, 항돌연변이, 항산화, 혈전용해, 항비만 활성, ACE 저해 효과 등 다양한 기능성 갖게 된다. 된장은 이처럼 우수한 건강 기능성 물질 함유하고 있는 반면, 인체에 해로운 aflatoxin 및 biogenic amine과 같은 유해물질 또한 포함 하는 것으로 알려져 전통된장의 우수성을 알리는데 걸림돌이 되고 있다. 따라서, 된장의 특성 및 발효과정에 관여하는

미생물 군집 분석과 그들의 역할을 확인하는 것은 된장의 발효특성을 파악하고 고품질의 장류를 제조하는데 매우 중요하다.

본 연구에서는 한국의 전통 콩발효식품인 메주와 된장의 미생물과 특성 분석 그리고 콩발효식품에 중요한 역할을 한다고 알려진 *Bacillus subtilis*의 지놈 분석을 실시하였다. 실험에 앞서 chapter 1에서는 메주와 된장에서의 최근 연구동향 및 그들의 기능적 특성에 대해 조사하였다.

2장에서는 시판된장의 미생물 군집분석 및 바이오제닉아민 함량을 분석하고 된장제조에 사용 가능한 종균 선발을 위해 된장으로부터 균주 분리 및 분리 균주의 안전성을 확인하였다. 순창지역에서 전통방식으로 제조된 재래식 된장 10개와 상업용으로 제조된 시판 된장 3개를 구매하여 실험을 수행하였다. 파이로시퀀싱 분석 기술을 이용하여 전통된장과 상업용 된장의 미생물 군집을 속(genus) 수준에서 확인한 결과 *Bacillus, Pseudomonas, Enterococcus, Staphylococcu* 종(species)이 우점종으로 확인되었고 *Wissella, Lactobacillus, Leuconostoc, Tetragenococcus*와 같은 LAB 등 매우 다양한 미생물이 존재하는 것을 확인할 수 있었다. 전통방식으로 제조된 된장에 비해 상업용 된장의 미생물 군집이 좀 더 단순한 것으로 확인되었지만 PCA 분석 결과 재래식 된장과 상업용 된장의 미생물 분포가 구분되지 않았다. 된장에

가장 많이 함유된 바이오제닉아민은 푸트레신(27.0–821.3 mg/kg), 2-페닐알라닌(4.1-2259.1 mg/kg), 히스타민(0-547.5 mg/kg), 티라민(0-657.3 mg/kg)으로 확인되었으나 된장에 따라 큰 차이를 보였다. 특히, 상업용 된장의 경우 모든 시료에서 스페르민, 히스타민, 티라민이 검출되지 않았으며 다른 바이오제닉아민 역시 재래식 된장에 비해 함량이 확연히 낮은 것을 밝혀냈다. 된장 종균을 선발하기 위해 히스타민의 함량을 기준으로 고히스타민 함유 된장 3개와 저히스타민 함유 된장 3개로부터 총 432 균주를 분리 동정하였다. 최종 염농도를 5%, 10%, 15%로 맞춘 TSA 이용하여 균주 분리를 진행한 결과 된장에서 *Bacillus licheniformis, Bacillus siamensis, Bacillus sonorensis, B. subtilis* subsp. *subtilis*가 우점하는 것을 확인하였으며, 염농도가 5%, 10%인 배지에서는 대부분 *Bacillus*가 분리되었지만, 15% 배지에서는 *Tetragenococcus*가 주로 분리되었다. 된장의 염농도가 15~18%인 것을 고려할 때 된장 내에서 *Bacillus*는 포자형태로 존재하며 *Tetragenococcus*가 발효를 이끌어 가는 역할을 담당할 것으로 예측된다. 분리된 432 균주 중 *Bacillus* 속 3 종과 *Oceanobacillus, Tetragenococcus, Virgibacillus* 속에 해당하는 균주를 대상으로 된장의 주 원료인 콩을 분해하기 위해 필요한 amylase, protease, lipase 활성을 5% NaCl이 첨가된 TSA에서 확인하였다. *B. siamensis*와 *B.*

subtilis 균주가 이들 중 세가지 효소에 대해 높은 활성을 보이는 반면, 다른 종들은 lipase에 대해서만 활성을 보였다. 이들 균주 중 식약처에 식품첨가제로 허가 되어있는 *B. subtilis* 47 균주를 대상으로 혈전분해능을 확인하였고 분해능이 우수한 12균주를 대상으로 균주의 특성 및 안전성을 검증하였다. 실험결과를 종합하여 amylase와 lipase 효소 활성이 우수하며 독성 및 항생제 내성을 갖지 않고 바이오제닉아민 생성능이 없는 *B. subtilis* 10TDI13균주를 된장 종균 후보로 선발하였다.

3장에서는 된장의 우점종으로 알려진 *B. subtilis*의 판지놈 분석을 수행하였다. *Bacillus*는 16S rRNA 유전자가 매우 잘 보존되어 있어 이를 이용해 *Bacillus*를 구분하는 것은 사실상 불가능하다. 따라서 본 연구에서는 ANI value와 in silico DDH value를 통해 *B. subtilis* genome을 계통학적 관점에서 다시 분류하고 각 그룹을 구분할 수 있는 unique gene 찾아내고자 하였다. 2장에서 분리한 *B. subtilis* 10TDI13를 포함한 모든 *B. subtilis* 지놈을 NCBI에서 다운받아 분석에 이용하였으나 지놈의 정보가 부족하거나 BPGA 프로그램 오류 야기, CheckM 결과 contamination 5% 이상을 보이는 지놈은 제거하고 최종 99 균주를 분석에 사용하였다. *B. subtilis*와 *Bacillus* group에 속하는 16 종의 표준균주 16S rRNA를 이용하여 phylogenetic tree를 그린 경우 대부분의 균주가 같은 clade에 존재함으로써

B. subtilis 아종(subspecies)은 물론 종의 구분이 불가능 한 것을 확인하였다. 하지만, *B. subtilis* 99 지놈을 이용하여 ANI value를 수행한 결과 *B. subtilis* 10TDI13은 *B. subtilis* subsp. *subtilis* (98.74-98.34%), *B. subtilis* subsp. *inaquosorum* (92.96-92.84%), *B. subtilis* subsp. *spizizenii* (93.04-93.04%)로 확인되었다. 따라서, 현재 *B. subtilis*에 속하는 3개의 아종은 서로 다른 종일 것으로 추측된다. 뿐만 아니라 ANI value를 통한 phylogeny tree 작성 시 기존 3개의 아종을 포함하여 총 8개의 clade로 나뉘는 것을 확인하였다. 이 같은 결과는 *in silico* DDH 및 308개의 core gene을 이용해 그린 phylogenetic tree 결과와도 일치하였다. 8개의 그룹 중 3개의 그룹은 *Bacillus atrophaeus, Bacillus amlyloliquefaciens, Bacillus velezensis*로 밝혀졌으며 분리된 균주의 동정 시 16S rRNA gene을 이용하는 과정에서 잘못 동정되거나 종이 재분류 될 때 생긴 오류일 것으로 판단된다. *B. subtilis*로 분류된 5개의 그룹 중 genome 수가 1개인 (II) 그룹을 제외하고 나머지 4개 그룹을 이용해 pan-core gene 분석을 수행하였고 이들 그룹은 1163개의 core gene을 가지고 있는 것을 밝혔다. KEGG pathway 분석 시 90%의 metabolic pathway를 4개의 *Bacillus* group 모두 가져 그룹간에 큰 차이를 보이지 않았다. 하지만, 1, 3, 4, 5 그룹은 각각 72, 3, 2, 13의 unique

gene을 보유하였으며, 이 유전자를 marker gene으로 이용함으로써 각 그룹을 구분하는데 활용 가능할 것으로 판단된다.

4장에서는 안전하고 균일한 품질의 된장 제조를 위해 종균을 이용하여 재래식 방법으로 메주를 제조하고 발효시기별 미생물 군집 및 대사체 변화 양상 대해 연구하였다. 종균으로 사용한 *Aspergillus oryzae* SNU-HR는 상업용 종균으로부터 분리된 균주이며 aflatoxin과 cyclopiazonic acid (CPA)를 분비하지 않는 균주이다. *A. oryzae* SNU-HR의 첨가 유무에 따라 메주의 미생물 군집과 효소활성, 대사체 등 다방면에서 차이를 보였다. 파이로시퀀싱을 사용한 세균 및 곰팡이의 군집분석에서 대조구의 경우 *Bacillus*와 *Hanseniaspora*가 발효초기에 우점인 것으로 나타났고, *A. oryzae* SNU-HR 첨가 시에는 *Bacillus*와 *Aspergillus*가 우점하는 것으로 확인되었다. 세균 군집의 경우 대조군과 실험군에서 군집을 이루는 세균의 종류에는 큰 차이를 보이지 않았지만 상대적 비율에는 큰 차이를 보였다. 하지만, 곰팡이 군집의 경우 대조군과 실험군에 존재하는 곰팡이의 군집 구성에서 확연한 차이를 보였는데 *A. oryzae* SNU-HR를 첨가한 메주는 단순한 군집을 보이는 반면 대조군의 경우 다양한 곰팡이 군집이 존재하는 것을 알 수 있었다. *A. oryzae* SNU-HR를 첨가한 메주는 전 발효기간에 걸쳐 *Aspergillus*가 우점하는 반면 대조군에서는 발효

초기에 *Hanseniaspora, Mucor, Sordaria*가 우점을 차지하지만 발효가 진행되면서 *Aspergillus*, unclassified *microascaceae*, *Gibberella* 속이 우점을 차지하는 것으로 관찰되었다. *A. oryzae* SNU-HR의 첨가는 곰팡이의 군집에만 영향을 미치는 것이 아니라 세균의 군집에도 큰 영향을 미치는 것으로 확인되었다. 대조군의 경우 잠정적 병원성 균인 *Staphylococcus*가 발효초기부터 발효가 끝나는 시점까지 대략 8-35% 정도 유지되는 것으로 확인되었다. 반면 *A. oryzae* SNU-HR를 첨가한 경우 이들의 비율은 확연히 감소하는 양상을 보여 안전성을 확보한 된장의 제조 가능성을 보여주었다. 또한, *A. oryzae* SNU-HR를 첨가한 경우 메주의 초기 amylase 활성이 대조군에 비해 2배 가량 높았고 protease 활성 또한 대조군에 비해 높은 것을 확인할 수 있었으며, 그 결과 *A. oryzae* SNU-HR가 첨가된 메주의 alanine, asparagine, aspartate, glutamate, glutamine, glycine가 같은 기간 대비 더 많이 생성되었다. 결론적으로, *A. oryzae* SNU-HR 첨가로 인해 메주의 안전성을 확보를 기대할 수 있을 뿐만 아니라 메주의 발효기간 단축 가능성을 보여주었다.

주요어: 메주, 된장, 고초균, 판지놈, 누룩곰팡이, 군집분석, 대사체.

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